Hemmung der Neuraminidase

von *Streptococcus pneumoniae*

im Synergismus mit Influenza-A-Viren

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Abkürzungen

A(H1N1)pdm09	Influenza-A-Viren der Pandemie 2009
Cbp	cholin-gebundene Proteine (engl. *cholin binding protein*)
CL-Test	chemilumineszenz-basierter Neuraminidase-Hemmtest
C. perfringens	*Clostridium perfringens*
DANA	N-Acetyl-2,3-Dehydro-2-Deoxyneuraminsäure
FL-Test	fluoreszenz-basierter Neuraminidase-Hemmtest
HA	Hämagglutinin
HA-HT	Hämagglutinations-Hemmtest
IC_{50}	mittlere inhibitorische Konzentration
Jena/8178	Influenzavirus A/Jena/8178/2009
M2	Matrixprotein 2
MHK *(MIC)*	minimale Hemmkonzentration (engl. *minimal inhibitory concentration*)
NA	Neuraminidase/n
NAI	Neuraminidase-Inhibitor/en
NanA	Neuraminidase A der Pneumokokken
NanB	Neuraminidase B der Pneumokokken
NanC	Neuraminidase C der Pneumokokken
Neu5Ac	N-Acetylneuraminsäure
NP	Nukleoprotein
PspA	Oberflächenprotein A der Pneumokokken
rNanA	rekombinante Neuraminidase A
rNanB	rekombinante Neuraminidase B
RNP	Ribonukleoprotein
S. pneumoniae	*Streptococcus pneumoniae*
STIKO	Ständige Impfkommission am Robert Koch-Institut

1 Einleitung

In 2009 a deadly virus burned through our civilization, pushing humankind to the edge of extinction. Dr. Robert Neville dedicated his life to the discovery of a cure, and the restoration of humanity. (I Am Legend, US-amerikanischer Film von 2007)

Der Film „I Am Legend“ von 2007 basiert auf dem Science-Fiction-Roman aus dem Jahre 1954 von Richard Matheson. Ein mutiertes Masern-Virus bedroht die menschliche Bevölkerung. Dem Virologen Dr. Robert Neville gelingt es, ein Gegenmittel zu entwickeln und die Menschheit zu retten. Die hohen Morbiditäts- und Mortalitätsraten werden im Film durch ein Virus verursacht, das schnell mutiert und mit anderen Pathogenen in Wechselwirkungen tritt. Diese Eigenschaften treffen auch auf das Influenzavirus („Grippevirus“) zu, welches jährlich zahlreiche Todesfälle fordert (Dawood *et al.*, 2012; Kasowski *et al.*, 2011). Nach Sekundärinfektion mit *Streptococcus (S.) pneumoniae* (auch Pneumokokken) führt Influenza vermehrt zu Todesfällen, was als letaler Synergismus bezeichnet wird.

1.1 Bedeutung von Pneumokokken

Charakterisierung und Epidemiologie

Weltweit erkranken jährlich 100 Millionen Kinder an Mittelohrentzündung, 5 Millionen Menschen an Lungenentzündung sowie 100.000 an Meningitis (Varon *et al.*, 2010). Als Hauptursache dieser Krankheiten sind Bakterien wie *S. pneumoniae* zu nennen (Mehr *et al.*, 2012), welche der Gruppe *S. mitis* der Gattung *Streptococcus* zugeordnet werden (Facklam, 2002). Der Durchmesser der grampositiven α-hämolysierenden Streptokokken beträgt 1–2 µm. Entdeckt wurden die Pneumokokken vor 130 Jahren von George Miller Sternberg in den USA und Louis Pasteur in Frankreich (Austrian, 1981). Das Genom besteht aus ca. 2,2 Millionen Basenpaaren mit etwa 2.000 Genen (Henriques-Normark *et al.*, 2013; Tettelin *et al.*, 2001).

Die aerotoleranten katalasenegativen Pneumokokken können bei invasiven Erkrankungen in den Blutkreislauf eindringen und so eine Sepsis verursachen (O'Brien *et al.*, 2009). *S. pneumoniae* gehört zur normalen Mikroflora des Menschen und besiedelt bis zu 80 % des Nasenrachenraums im Kindesalter (Bogaert *et al.*, 2004; Cardozo *et al.*, 2008). Im Alter von 10 Jahren sinkt die Prävalenz der Kommensale auf 8–12 % und beim Erwachsenen weiter auf 3–4 %. Laut Statistik erkrankt jedes Kind einmal pro Jahr an einer Mittelohrentzündung (DGPI, 2009), die zu 38 % von Pneumokokken verursacht wird. Schätzungsweise

kommt es in Deutschland durch Pneumokokken jährlich zu 8.000 bis 12.000 Todesfällen, vor allem bei Menschen über 65 Jahre oder bei Patienten mit bestehenden Vorerkrankungen (Hülße *et al.*, 2011).

Nahezu alle *S. pneumoniae*-Isolate, die Infektionen verursachen, sind von einer Polysaccharidkapsel umgeben. Aufgrund der chemischen Zusammensetzung der Kapsel und der daraus folgenden Antigenität unterscheiden Bakteriologen über 90 Serotypen (Henriques-Normark *et al.*, 2013; Song *et al.*, 2013). Diese werden anhand ihrer immunologischen Ähnlichkeiten zu 46 Serogruppen zusammengefasst (Hausdorff *et al.*, 2005). In Deutschland gehören die Serotypen 1, 3, 4, 6A, 7F, 14, 19A, 22F und 23F zu den am weitesten verbreiteten. Besonders virulent verhält sich dabei der Serotyp 3 (Hülße *et al.*, 2011). Die Besiedlung gilt als Voraussetzung für eine invasive Erkrankung, welche vermehrt bei Kleinkindern auftritt (Gray *et al.*, 1980). Die Begründung liegt in der geringen humoralen Immunantwort gegen die Polysaccharidkapsel und in einer erhöhten Prädisposition gegenüber viralen Erkrankungen (Hausdorff *et al.*, 2005; Klugman *et al.*, 2009). Ähnliches gilt für ältere Menschen. Darüber hinaus führt ein Kontakt zu den Enkelkindern oft zu einer Übertragung, da der Nasenrachenraum der Kinder mit Bakterien besiedelt ist (Hausdorff *et al.*, 2005). Die Verteilung der Serotypen variiert je nach Alter, Krankheit und geografischer Verbreitung (ECDC, 2013).

Virulenzfaktoren

Die Kapsel stellt die wichtigste Determinante der Virulenz dar, da sie die Pneumokokken vor Phagozytose schützt (Magee *et al.*, 2001; Mitchell *et al.*, 2010). Bei einer reduzierten Kapselexpression erkennen Antikörper und Komplementsystem die Bakterien effektiver, was eine verstärkte Eliminierung durch das Immunsystem des Wirtes zur Folge hat (Hyams *et al.*, 2010; Nelson *et al.*, 2007). Die Zellwand unter der Kapsel, bestehend aus Polysacchariden und Teichonsäuren, fördert entzündliche Prozesse, wie die Aktivierung der Komplementkaskade und der Zytokinproduktion (Ghasemi *et al.*, 2009). Weiterhin dient sie als Anker für zellwand-assoziierte Proteine. Diese werden in peptidoglykan-gebundene Proteine (Hyaluronat-Lyase, Neuraminidasen (NA)), cholin-gebundene Proteine (CbpA, PspA, Autolysin) und zytoplasmatische, an die Lipiddoppelschicht gebundene Makromoleküle (PsaA) unterteilt (Jedrzejas, 2004; Paton *et al.*, 1993). Aus vorangegangenen Studien folgt, dass diese Proteine bei invasiven Erkrankungen hochreguliert werden (LeMessurier *et al.*, 2006; Orihuela *et al.*, 2004). In Tierversuchen zeigen entsprechende

Deletionsmutanten keine Virulenz mehr (Berry *et al.*, 2000; Jedrzejas, 2001; McDaniel *et al.*, 1987). Als wichtigster intrazellulärer Virulenzfaktor, der von allen Isolaten gebildet wird, gilt das porenbildende Pneumolysin. Das Toxin wird bei der Autolyse durch das Autolysin LytA freigesetzt und interagiert mit dem Cholesterol der Wirtszellmembran, was zum Verlust der Membranintegrität führt (Berry *et al.*, 1989; Berry *et al.*, 1999; Wellmer *et al.*, 2002). Zudem inhibiert es die Zilienbewegung und erhöht so die Pneumokokken-Migration (Marriott *et al.*, 2008).

1.2 Die Influenzaviren

Die Influenzaviren gehören zur Familie der Orthomyxoviridae und werden in sechs Genera (Influenza-A-, B- und C-, Thogoto-, Quaranja- und Isavirus) eingeteilt (Fauquet *et al.*, 2005; ICTV, 2014). Die pleomorphen Influenza-A- und -B-Viren besitzen ein Einzelstrang-RNA-Genom in Negativ-Orientierung mit acht Segmenten. Die Virushülle der Influenza-A-Viren besteht aus der Wirtszellmembran, in der das Hämagglutinin (HA), die NA und das Matrixprotein 2 (M2) mit ihren hydrophoben Domänen verankert sind (Das *et al.*, 2010; Gamblin *et al.*, 2010). Das Matrixprotein 1 kleidet die Virushülle von innen aus und stellt den Kontakt zu den Ribonukleoprotein-Komplexen (RNP-Komplexe) her (Martin *et al.*, 1991; Sha *et al.*, 1997). Die acht RNP-Komplexe bestehen jeweils aus einem Genomsegment, den Polymerase-Proteinen PB1, PB2 und PA und dem Nukleoprotein (NP) (Dias *et al.*, 2009; Portela *et al.*, 2002). Influenzaviren besitzen weitere Nichtstrukturproteine, z. B. NS1 und NS2/NEP (Fujii *et al.*, 2005). Neben den genannten zehn Proteinen (Palese *et al.*, 2007) wurden in den vergangenen Jahren weitere entdeckt: PB1-F2 (Chen *et al.*, 2001), PB1-N40 (Wise *et al.*, 2009), PA-X (Jagger *et al.*, 2012), PA-N155 und PA-N182 (Muramoto *et al.*, 2013).

Bei der Replikation binden die Virionen zunächst über das HA an α2,3- oder α2,6-verknüpfte N-Acetylneuraminsäuren (Sialinsäuren, Neu5Ac), die auf der Oberfläche der Wirtszelle sitzen. Dabei bevorzugen aviäre Viren die Bindung an α2,3- und humane Viren an α2,6-verlinkte Galaktose (Gamblin *et al.*, 2010). Auf den porzinen Atemwegsepithelien werden beide Rezeptorklassen exprimiert, daher zirkulieren sowohl avian-like als auch human-like HA-Stämme unter den porzinen Influenzaviren (Gambaryan *et al.*, 2005).

Über rezeptor-vermittelte Endozytose gelangt das Virion in die Zelle (Rossman *et al.*, 2011). Der geringe pH-Wert im Endosom führt zur Fusion von viraler und endosomaler Membran und zur Freisetzung der viralen RNP-Komplexe in das Zytoplasma. Dieser

Prozess wird durch eine Strukturänderung im HA induziert (Skehel *et al.*, 2000) und hängt darüber hinaus von der Ionenkanal-Aktivität des M2-Proteins ab (Pinto *et al.*, 1992). Die RNP-Komplexe werden in den Nukleus importiert, wo sie als Matrize für die Produktion der viralen mRNA fungieren (Cros *et al.*, 2003). Das virale Genom wird über ein Zwischenprodukt (cRNA) mittels der Polymerase-Proteine repliziert.
Im Anschluss erfolgt der Export der mRNA aus dem Nukleus, die Translation der membran-assoziierten Proteine HA, NA und M2 an der Membran des rauen endoplasmatischen Retikulums sowie deren Transport über den zellulären Endozytoseapparat zur Zelloberfläche. Während des Transports werden die Proteine glykosyliert, acetyliert und im Fall des HA durch zelluläre Proteasen in die Untereinheiten HA1 und HA2 gespalten (Wiley *et al.*, 1987). Die übrigen Proteine werden aus den Primärtranskripten translatiert, wandern durch die Exportfunktion der NS2/NEP-Proteine ins Zytoplasma ein und werden an jene Stellen transportiert, wo erhöhte Konzentrationen an HA, NA und M2 in der Zellmembran auftreten. Nun schließt sich der Buddingprozess, die Freisetzung der neugebildeten Viruspartikel, an. Der finale Schritt der Replikation obliegt der viralen NA, die die Sialinsäuren abschneidet und so die Virionen freisetzt (Nayak *et al.*, 2009).

1.3 Der letale Synergismus von Influenzaviren und Pneumokokken

Einfluss der bakteriellen Sekundärinfektion auf Influenza-Infektionen

Welchen fatalen Einfluss Sekundärinfektionen mit Pneumokokken auf eine Influenza-Infektion haben, wurde retrospektiv durch die Spanische Grippe von 1918 gezeigt (Morens *et al.*, 2008). Bei der Pandemie infizierte sich ein Drittel der Weltbevölkerung mit Influenza-A-Viren vom Subtyp H1N1 was zum Tod von 40 bis 50 Millionen Menschen führte (Taubenberger *et al.*, 2006). In etwa 95 % der Todesfälle waren bakterielle Sekundärinfektionen, meist *S. pneumoniae* oder *Staphylococcus aureus* die Ursache (Kuiken *et al.*, 2008; Morens *et al.*, 2008). Weitere Pandemien mit bakteriellen Einflüssen folgten: die Asien-H2N2-Grippe (1957–1958) und die Hongkong-H3N2-Grippe (1968–1969). In der Schweinegrippesaison von 2009 lag die Mortalität ähnlich hoch wie bei der saisonalen Influenza und der Anteil der bakteriellen Sekundärinfektionen betrug 25–50 % (Dawood *et al.*, 2012; Gill *et al.*, 2010; Pavia, 2013). Generell variiert die Zahl der Krankheits- und Sterbefälle von Jahr zu Jahr, mit tendenziell häufigeren Krankenhausaufenthalten bei Epidemien mit zirkulierenden H3N2-Viren (McCullers, 2006a). Wie viele Menschen während einer Pandemie an bakteriellen Sekundärinfektionen genau versterben, lässt sich nur schwer erfas-

sen. Gründe sind zum einen die geringe Sensitivität und Spezifität der Diagnostik (Nolte, 2008). Zum anderen kann das Virus bereits vom Körper eliminiert worden sein, wenn die Bakterien detektiert werden, wodurch sich kein Zusammenhang zur Pandemie mehr herstellen lässt (McCullers, 2014).

Viraler Einfluss auf die Pneumokokken-Infektion

Eine Vielzahl von Mechanismen begünstigt die bakterielle Ausbreitung im Respirationstrakt nach einer Virusinfektion (Bosch *et al.*, 2013; Grijalva *et al.*, 2012; McCullers, 2006b). Mit Hilfe von *In-vivo*-Modellen mit einem hochpathogenen Influenzavirusisolat wurde die Zerstörung der Epithelzellen als eine Hauptursache herausgestellt (Harford *et al.*, 1949; McCullers *et al.*, 2001; McCullers *et al.*, 2002). Durch den Gewebeschaden ergeben sich zahlreiche Bindungsstellen und neue Nährstoffquellen für die Pneumokokken. Dies zeigt sich in beschleunigtem Bakterienwachstum und erhöhten bakteriellen Titern in der Lunge. Die Freisetzung von fibrinogenem Material und Muzinen führt zur Blockierung der kleinen Luftwege und so zur reduzierten Sauerstoff- und Kohlendioxiddiffusion (Horner *et al.*, 1973). Es entsteht eine sogenannte „Todeszone“, in der die Zilienbewegung vermindert und unkoordiniert ist (Levandowski *et al.*, 1985). Als Folge bleibt die mechanische Beseitigung der Bakterien aus, was vor allem bei Patienten mit chronischen Erkrankungen zu Problemen führt (Glezen *et al.*, 2000). Allerdings konnten andere Forschungsgruppen nach Infektion mit niedrigpathogenen Viren, die leichte Gewebeschäden in der Lunge verursachen, auch eine verlängerte Persistenz der Pneumokokken im Vergleich zur Verweildauer ohne vorherige Virusbehandlung beobachten (Alymova *et al.*, 2005; Harford *et al.*, 1950; McCullers *et al.*, 2002). Durch die Zerstörung der Epithelien entstehen Rezeptorbindestellen für Virulenzfaktoren wie Cbp und PspA (Plotkowski *et al.*, 1986). Die virale NA spaltet Sialinsäuren ab, wodurch kryptische Rezeptoren für die Pneumokokken und die Sialinsäuren als Nahrungsquelle entstehen (Siegel *et al.*, 2014; Tong *et al.*, 1999; Tong *et al.*, 2002).

Weiterhin können Polymorphismen in der Aminosäuresequenz des HA den Tropismus von Influenza-A-Viren verändern und so auch die Kopathogenese mit *S. pneumoniae* im oberen und unteren Respirationstrakt beeinflussen (McCullers, 2014). Das HA der pandemischen H1N1-Influenza-A-Viren von 2009 (A(H1N1)pdm09) bindet bevorzugt an α2,6-gebundene Sialinsäuren auf den Wirtsepithelzellen des oberen Respirationstrakts (Chutinimitkul *et al.*, 2010; Seidel *et al.*, 2014). Aus diesem Grund replizieren A(H1N1)pdm09 eher im

oberen Respirationstrakt und auch die Koinfektion findet bevorzugt dort statt (McCullers, 2014). Allerdings kann es bei den Influenzaviren durch den genetischen Drift (Mutation einzelner Aminosäuren) und den Antigenshift (Reassortierung ganzer Gensegmente) zu veränderten Bindungspräferenzen des HA kommen. Des Weiteren spielt der Glykosylierungsstatus des HA eine große Rolle. Wirtslektine binden hochglykosylierte Viren, sodass diese schnell eliminiert werden (Vigerust *et al.*, 2007). Das HA des Virus von 1918 verfügte nur über eine geringe Glykosylierung und band an α2,3-verknüpfte Sialinsäuren (McCullers, 2014). Diese Kombination verursachte die schweren Komplikationen mit Todesfolge.

Die Entzündungsreaktion nach einer Virusinfektion begünstigt auch die Infektion mit Pneumokokken (Metzger *et al.*, 2013; Short *et al.*, 2012; Sun *et al.*, 2008). So beeinträchtigt Interferon α und γ sowie Interleukin 10 die Immunantwort auf die Bakterien (Navarini *et al.*, 2006; van der Sluijs *et al.*, 2006). Interleukin 10 wird bei der Erkennung der viralen Nichtstrukturproteine durch Toll-like-Rezeptoren produziert und unterdrückt die Makrophagen- und Neutrophilantwort, die im Normalfall zur Beseitigung der Bakterien führen würden (Didierlaurent *et al.*, 2008). Das virale proapoptotische Protein PB1-F2 verursacht Entzündungsreaktionen, welche sich als erhöhte zelluläre Infiltration in Lunge und Atemwegen sowie in einem Zytokinsturm manifestieren (McAuley *et al.*, 2007). Die überschießende Immunantwort wird durch frei werdende Entzündungsmediatoren bei der Lyse der Bakterien weiter gefördert (McCullers, 2014). Eine effektive Pathogenkontrolle bleibt aus. Bei der verzögerten Wundheilung entstehen neue bakterielle Bindungsstellen an Laminin, Kollagen und Fibronektin, was wiederum die Manifestation der Bakterien im Respirationstrakt verlängert (Martin *et al.*, 2005).

Synergistische Effekte treten vor allem dann auf, wenn die Viruserkrankung der bakteriellen Infektion vorausgeht (Huber *et al.*, 2010; McCullers *et al.*, 2001; McCullers *et al.*, 2002; McCullers, 2006b; McCullers *et al.*, 2010). Dabei wirken sich die Pneumokokken am stärksten am 7. Tag nach der Virusinfektion aus, wenn der Wirt das Virus bereits eliminiert hat (McCullers, 2006b).

Einfluss von* S. pneumoniae *auf die Infektion mit Influenza-A-Viren

Auch bakterielle Mechanismen können die Virusreplikation beeinflussen, jedoch fehlt es noch an Untersuchungen. Der Virustiter der Maus ist nach einer *S. pneumoniae*-Infektion stark erhöht (McCullers *et al.*, 2002). Eine Erklärung für diesen Anstieg und den damit

verbundenen Gewebeschaden in der Lunge sind bakterielle Proteasen, die die Spaltung des HA-Vorläuferprotein HA0 in seine aktiven Komponenten HA1 und HA2 fördern (Tashiro *et al.*, 1987). Ein weiterer Einflussfaktor könnte die bakterielle NA sein, die den Virusertrag steigert, indem sie die Funktion der viralen NA unterstützt (Nishikawa *et al.*, 2012). Die Immunantwort spielt auch bei der bakteriellen Infektion eine entscheidende Rolle. So fördert die Zellwand oder das Pneumolysin die Entzündungsreaktion (Kadioglu *et al.*, 2004).

1.4 Prophylaxe und Therapie der Influenza- und Pneumokokken-Infektion

Pneumokokken und Influenzaviren gehören zu den wichtigsten humanen Pathogenen des Respirationstraktes. Es existieren für beide Mikroorganismen Schutzimpfungen. Jedoch stufen Ärzte die Impfraten für die Influenza in Deutschland als zu niedrig ein (Gesundheit in Deutschland aktuell (GEDA), 2012). In der Saison 2009/10 waren einer Studie zufolge lediglich 27 % des medizinischen Personals gegen saisonale Influenza geimpft (Böhmer *et al.*, 2012). Für ältere Menschen hat die Weltgesundheitsorganisation eine Impfrate von 75 % als Ziel gesetzt (WHO, 2005a). Daten aus „Gesundheit in Deutschland aktuell" zeigen, dass die Impfrate in dieser Bevölkerungsgruppe in Deutschland bei 53 % im Jahr 2012 lag.

Pneumokokken-Impfung

Durch die 90 Serotypen gestaltet sich die Impfstoffzusammensetzung im Falle der Pneumokokken schwierig. Von der Ständigen Impfkommission am Robert Koch-Institut (STIKO) wird die Schutzimpfung für Säuglinge und Kleinkinder zwischen 2 und 23 Monaten sowie für Menschen ab 60 Jahren empfohlen. Es existieren zwei Impfstoffe: zum einen der Konjugatimpfstoff (10- bzw. 13-valent) für Säuglinge und Kleinkinder und zum anderen der Polysaccharidimpfstoff (23-valent) für Erwachsene. Synflorix® (Serotypen: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F und 23F) ist seit April 2009 für Säuglinge bis 23 Monate zugelassen und löst den 7-valenten Impfstoff Prevenar® ab (Knuf *et al.*, 2010). Prevenar13® (seit Ende 2009) beinhaltet die Serotypen 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F und 23F, wird bis zum vollendeten 5. Lebensjahr geimpft und deckt 84 % der zirkulierenden Serotypen ab (Duggan, 2010). Bei beiden Impfstoffen sind die einzelnen Kapselantigene an ein Trägerprotein gekoppelt, welches die T-Zell-abhängige Immunantwort induziert und so Gedächtniszellen entstehen lässt (Pletz *et al.*, 2008). Die einmalige Impfdosis für Erwachsene (Pneumovax®) enthält hochgereinigte Kapselpolysaccharide der

23 Pneumokokken-Typen, die etwa 90 % aller systemischen Pneumokokken-Erkrankungen verursachen (Hülße *et al.*, 2011). Allerdings verhindert Pneumovax® nicht die Infekte der oberen Atemwege. Seit Einführung der generellen Impfempfehlung beobachten Wissenschaftler eine 50%ige Abnahme der Fallmeldungen bei den unter Zweijährigen (Rückinger *et al.*, 2009). Allerdings nehmen seit einigen Jahren die Erkrankungsraten wieder zu, da sich Serotypen ausbreiten, die nicht durch die Impfung abgedeckt sind (Mehr *et al.*, 2012). Daher sollte die Erforschung alternativer Impfstrategien weiter in den Fokus rücken (Adamou *et al.*, 2001; Talkington *et al.*, 1996; Tong *et al.*, 2005; Wizemann *et al.*, 2001).

Influenza-Impfung

Die hohe Mutationsrate des Influenzavirus (genetischer Drift) erfordert jedes Jahr die Herstellung und Verabreichung eines neuen Impfstoffs. Der aktuelle tri- und quadrivalente Totimpfstoff von 2015 enthält Antigene eines A(H1N1)pdm09 (A/California/07/2009), eines H3N2-Virus (A/Texas/50/2012) und eines Influenza-B-Virus (B/Massachusetts/2/2012). Der quadrivalente Impfstoff beinhaltet zusätzlich Antigene des Influenzavirusstammes B/Brisbane/60/2008 (Buda *et al.*, 2015). In der Influenzasaison 2014/15 lag der Anteil von Influenza-A-Viren vom Typ H3N2 bei 76 %, von A(H1N1)pdm09-Viren bei 14 % und von Influenza-B-Viren bei 8 % (Robert Koch-Institut, Stand Woche 12/2015, Buda *et al.*, 2015). Allerdings reagierte das zirkulierende Influenza-A-Virus (H3N2) nur vermindert mit dem Immunserum des Impfstammes A/Texas/50/2012, wodurch eine starke Grippewelle mit 181 Todesfällen in Deutschland zu verzeichnen war (Robert Koch-Institut, Stand Woche 12/2015).

Impferfolge der Ko-Immunisierung

Zahlreiche Studien belegen, dass die Influenza-Impfung vor der bakteriellen Sekundärinfektion schützt (Huber *et al.*, 2010; Sun *et al.*, 2011). Bei Kindern verringert die Virusimpfung während eines Influenza-Ausbruchs das Auftreten von Mittelohrentzündungen um ca. 30 % (Belshe *et al.*, 1998; Heikkinen *et al.*, 1991). Auch bei Älteren ist ein Effekt auf die Mortalität bei der ambulant erworbenen Pneumonie zu verzeichnen (Nordin *et al.*, 2001). Die Pneumokokken-Impfung wirkt sich auch auf die influenzabedingte Pneumonie aus (Grijalva *et al.*, 2012; Klugman *et al.*, 2009; Madhi *et al.*, 2004). Bei der Immunisierung mit beiden Impfstoffen werden nosokomiale Pneumonien und invasive Pneumokokken-Erkrankungen weiter reduziert (Hedlund *et al.*, 2003). Aufgrund der hohen Mutationsrate und

Variabilität der Virulenzfaktoren beider Pathogene erscheint jedoch eine Alternative zur Impfung unabdingbar. Chronische und immungeschwächte Patienten sowie Kleinkinder können zudem oft nicht geimpft werden und sind auf die Therapie angewiesen (van der Poll *et al.*, 2009).

Chemotherapie der Pneumokokken

In der Regel erfolgt die Therapie der Pneumokokken-Infektionen mit Antibiotika, meist mit Penicillinen oder Makroliden (ECDC, 2013). Jedoch beobachtet die STIKO weltweit seit Jahren die Zunahme an antibiotika-resistenten Stämmen (Reinert, 2002; STIKO, 2006). Im Vergleich zu Frankreich, Polen und Spanien (ca. 25 %) liegt die Rate der Penicillinresistenz in Deutschland bei 5–10 %, allerdings mit zunehmender Tendenz (ECDC, 2013). Die Makrolidresistenz stieg dagegen bereits auf 10–25 %. Durch die natürliche Kompetenzfähigkeit der Pneumokokken können Resistenzgene von verschiedenen Streptokokkenstämmen übertragen werden (Moscoso *et al.*, 2004). In Tiermodellen wurde nachgewiesen, dass β-Laktamantibiotika wie Ampicillin zusätzlich zu Oseltamivir die Krankheitsdauer einer Pneumonie nach einer Influenzaerkrankung verkürzen (McCullers, 2004).

Chemotherapie der Influenza

Im Replikationszyklus der Influenzaviren existieren verschiedene Angriffspunkte für eine Therapie (Das *et al.*, 2010; Du *et al.*, 2012). Als erstes Zielprotein wurde das Ionenkanalprotein M2 identifiziert und die Medikamente Amantadin und Rimantadin entwickelt (Hay *et al.*, 1985). Jedoch entstanden Resistenzen gegen diese M2-Blocker (Monto *et al.*, 1992; Schmidtke *et al.*, 2005). In der Saison 2005/06 waren über 90 % der H3N2-Isolate und die Hälfte der H1N1-Isolate durch eine Substitution S31N im M2-Protonenkanal gegen Amantadin unempfindlich (Deyde *et al.*, 2007; Gubareva *et al.*, 2010; Schmidtke *et al.*, 2008). Derzeit tragen nahezu alle zirkulierenden Viren Mutationen, die Amantadin-Resistenzen vermitteln (CDC, 2011).

Zur Influenza-Therapie stehen momentan nur NA-Inhibitoren (NAI) zur Verfügung, die in dieser Arbeit im Fokus stehen. Mit der Aufklärung der dreidimensionalen Struktur der viralen NA wurde gezeigt, dass das aktive Zentrum von allen Influenza-A- und -B-Stämmen eine hohe Bindungsspezifität für neuraminsäurehaltige Strukturen besitzt (von Itzstein *et al.*, 1993). NAI interagieren direkt mit diesem aktiven Zentrum, hemmen kompetitiv die Funktion des Enzyms und somit die Virusfreisetzung. Infolgedessen kommt es zur Aggre-

gation der Viruspartikel auf der Zelloberfläche und die Virusausbreitung wird inhibiert (Palese, 2004). Zurzeit werden Oseltamivir (Tamiflu®, orale Applikation) und Zanamivir (Relenza®, inhalative Applikation) in der Therapie in Deutschland eingesetzt. Im Oktober 2009 hat die US-amerikanische Zulassungsbehörde FDA (Food and Drug Administration) einen weiteren NAI, Peramivir (Rapivab®/Rapiacta®, intravenöse Applikation), zur Notfallbehandlung für hospitalisierte Patienten mit Verdacht auf Schweinegrippe zugelassen (Birnkrant *et al.*, 2009). Peramivir wird seit 2010 in Japan und Südkorea sowie seit Dezember 2014 in den USA bei der Influenzabehandlung angewendet (de Jong *et al.*, 2014; Leang *et al.*, 2014). Laninamivir (Inavir®, inhalative Applikation) wird als weiterer NAI in Japan in der Influenza-Therapie erfolgreich eingesetzt (Leang *et al.*, 2014). In der Saison 2007/08 traten erstmalig oseltamivir-resistente H1N1-Stämme mit dem Aminosäureaustausch H274Y auf (Meijer *et al.*, 2009). NAI-Resistenzen werden außerdem durch Substitutionen an den Aminosäurepositionen Y155, I222, E119 und N294 (N2-Nummerierung) vermittelt (Grienke *et al.*, 2012; Nguyen *et al.*, 2012).

Neben den NAI gibt es NP-Inhibitoren wie Nukleozin, welches eine NP-Aggregation auslöst (Gerritz *et al.*, 2011; Kao *et al.*, 2010) und den Polymerase-Inhibitor Favipiravir (Furuta *et al.*, 2013). Letzterer ist in Japan bereits zur Behandlung von verschiedenen RNA-Viren zugelassen (Hurt *et al.*, 2015). Weitere Substanzen, die z. B. das HA blockieren, befinden sich in der Entwicklung (Russell *et al.*, 2008; Smee *et al.*, 2008).

1.5 Neuraminidasen als neues therapeutisches Target bei Pneumokokken?

Im Gegensatz zu den Influenzaviren exprimieren Pneumokokken bis zu drei verschiedene NA (Camara *et al.*, 1994), wobei NanA in allen Stämmen vorkommt, NanB in 96 % und NanC bei ca. der Hälfte der Isolate (Pettigrew *et al.*, 2006). Die Pneumokokken-NA agieren als Monomer, während die Influenza-NA als Tetramer vorliegt (von Grafenstein *et al.*, 2015). Obwohl sich die NA in der Aminosäuresequenz stark unterscheiden (Sequenzidentität < 10 % zwischen bakterieller und viraler NA), bestehen strukturelle und funktionelle Gemeinsamkeiten (von Grafenstein *et al.*, 2015). Zum Beispiel katalysieren alle NA die Spaltung von terminalen Sialinsäuren von verschiedenen Glykokonjugaten auf der Zelloberfläche (Taylor, 1996).

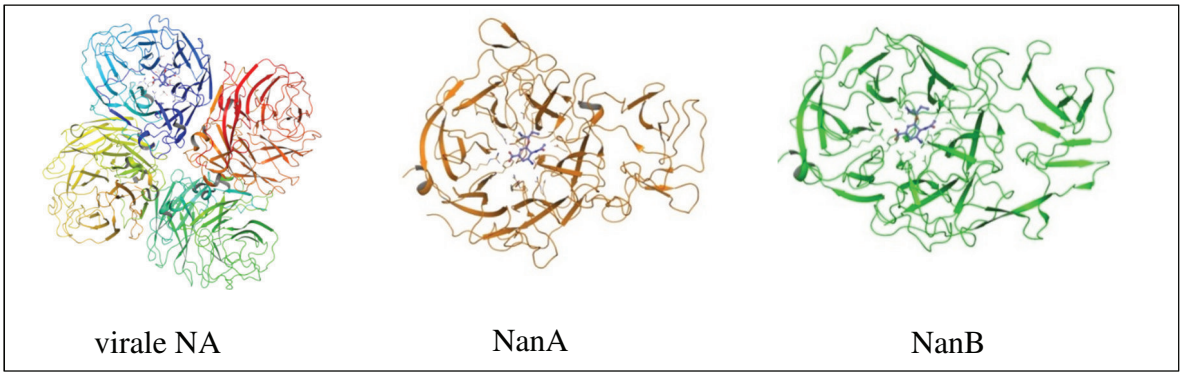

Abbildung 1: Röntgenkristallstruktur der viralen Neuraminidase (NA), Pneumokokken-NanA und -NanB (mit freundlicher Genehmigung von Prof. Dr. Johannes Kirchmair, Universität Hamburg, Zentrum für Bioinformatik).

NanA

Die NanA der Pneumokokken gilt als die am besten untersuchte NA und wird als wichtiger Virulenzfaktor beschrieben (King *et al.*, 2005). NanA besitzt ein Molekulargewicht von 108 kDa und besteht aus 1035 Aminosäuren. Über das LPxTG-Motiv am C-terminus ist die NanA in der Zellwand verankert (Camara *et al.*, 1994). Die Sialidase hydrolysiert α2,3-, α2,6- und α2,8-verknüpfte Sialinsäuren (Xu *et al.*, 2008a). Bei einer Enzymreaktion bindet der Carboxylsäurerest des Sialinsäuresubstrates in eine Tasche, die von einer Arginintriade im katalytischen Zentrum der NA gebildet wird (Xu *et al.*, 2008a). Diese Arginintriade ist bei viralen als auch bei bakteriellen NA an der enzymatischen Aktivität beteiligt. Das Kohlenhydrat-Bindemodul am N-Terminus trägt auch zur Spaltung von Sialoglykosiden bei, indem es die Bindung mit den Epithelzellen verstärkt (Uchiyama *et al.*, 2009).

Bei der Interaktion von Pneumokokken mit Wirtszellen oder freien Sialinsäuren tritt eine erhöhte Expression von NanA auf (LeMessurier *et al.*, 2006; Song *et al.*, 2008; Yesilkaya *et al.*, 2008). Zudem zeigen zahlreiche Studien, dass NanA bei der Kolonisation des Nasenrachenraumes und der Ausbreitung in der Lunge (Orihuela *et al.*, 2004; Trappetti *et al.*, 2009), bei der Sepsis (Manco *et al.*, 2006), als auch bei der Entstehung von Mittelohrentzündungen (Muñoz-Elías *et al.*, 2008; Tong *et al.*, 2000; Tong *et al.*, 2002) sowie des hämolytisch-urämischen Syndroms (Smith *et al.*, 2013) beteiligt ist. Weiterhin fördert NanA die Überwindung der Blut-Hirn-Schranke und somit den Beginn einer Meningitis (Banerjee *et al.*, 2010; Uchiyama *et al.*, 2009). Auch der Einfluss von NanA auf die Biofilmbildung der Pneumokokken wurde beschrieben (Brittan *et al.*, 2012; Oggioni *et al.*, 2006; Parker *et al.*, 2009; Trappetti *et al.*, 2009). Allerdings existieren auch Publikationen, die in ihren Experimenten einen sehr geringen oder keinen Effekt der NanA auf die Viru-

lenz der Pneumokokken nachweisen (King *et al.*, 2004; King *et al.*, 2006). So konnte in beiden Publikationen ein NanA-defizienter Pneumokokkenstamm den Nasenrachenraum von Mäusen und Ratten im gleichen Maß kolonisieren wie der Wildtyp. Weiterhin stimmte das Adhärenzverhalten der Mutante *in vitro* mit dem des Wildtyps überein (King *et al.*, 2006).

NanA trägt darüber hinaus zur Bereitstellung von Nährstoffen bei (Buckwalter *et al.*, 2012; Gualdi *et al.*, 2012; Marion *et al.*, 2011; Siegel *et al.*, 2014). Im Zusammenspiel mit anderen Oberflächenproteinen (BgaA, β-Galaktosidase und StrH) kommt es durch sequentielle Deglykosylierung von komplexen Kohlenhydraten wie Muzinen zur Freisetzung von Sialinsäuren, die als Kohlenstoff- und Energiequelle fungieren (Burnaugh *et al.*, 2008; King *et al.*, 2006; Limoli *et al.*, 2011). Zudem entstehen durch die Abspaltung kryptische Rezeptoren für die Anheftung weiterer Pneumokokken.

NanB

NanB ist mit 75 kDa etwas kleiner als NanA, besitzt keine Ankerdomäne und wird sezerniert (Berry *et al.*, 1996; Xu *et al.*, 2011). Als Trans-Sialidase überträgt NanB 2,7-Anhydro-Neu5Ac von bevorzugt α2,3-gespaltenen Sialinsäuren auf andere Glykokonjugate (Gut *et al.*, 2008; Xu *et al.*, 2008b). Manco *et al.* belegten, dass neben NanA-defizienten Pneumokokkenstämmen auch NanB-defiziente Isolate den Nasenrachenraum nicht kolonisieren und auch keine Sepsis mehr auslösen (Manco *et al.*, 2006). Die erhöhte Expression von NanB im Biofilm *in vitro*, als auch im Tiermodell der Pneumonie und Meningitis, wurde ebenso demonstriert wie die für NanA (Manco *et al.*, 2006; Oggioni *et al.*, 2006). Allerdings weisen die pH-Optima der Enzyme (pH 6,5–7,0 für NanA und pH 4,5–5,0 für NanB) sowie die verschiedenen Substratspezifitäten auf unterschiedliche Rollen in der Pathogenese der Bakterien hin (Berry *et al.*, 2000; Gut *et al.*, 2008; Xu *et al.*, 2011).

NanC

Zur Bedeutung der dritten NA NanC ist wenig bekannt. Da sie nur bei der Hälfte der Stämme exprimiert wird, kommt sie als therapeutisches Target nicht in Betracht. NanC scheint NanA und NanB im bakteriellen Metabolismus zu regulieren (Xu *et al.*, 2011).

Neue Ansätze zur Identifizierung von NAI

Im medizinischen Bereich gibt es einen großen Bedarf an neuen Influenza-Inhibitoren, da immer wieder Resistenzen auftreten (Chamni *et al.*, 2013). In den letzten Jahren, vor allem nach der Pandemie 2009, haben sich viele Forschungseinrichtungen die Identifizierung von

neuen Substanzen mit NA-inhibitorischen Potenzial als Ziel gesetzt (Grienke *et al.*, 2012). Mit Hilfe von Röntgenkristallstruktur-Analysen der NA und des katalytischen Zentrums im Speziellen können Computermodellierungen die Bindungseigenschaften von NAI voraussagen (Grienke *et al.*, 2013). Mit diesen Ergebnissen wird im Anschluss ein *In-silico*-Screening durchgeführt (Chamni *et al.*, 2013). Die Wirkung der Moleküle, die virtuell in die Bindetasche der NA passen, wird dann in target- und zellbasierten Assays geprüft (Grienke *et al.*, 2012). Enzym-Hemmtests, fluoreszenz- oder chemilumineszenz-basiert (FL- oder CL-Test), verdeutlichen die direkte Wirkung auf die NA. Als zellbasierter Assay wird häufig der zytopathische Effekt-Hemmtest verwendet (Schmidtke *et al.*, 2001).

Beim ethnopharmakologischen Ansatz versuchen Pharmazeuten, NAI aus Naturstoffen zu gewinnen. Dazu wird in Medizinbüchern oder Überlieferungen aus dem Volksmund nach Stichworten wie „Erkältung“, „Schnupfen“ oder ähnlichem gesucht. Beispielswiese wurde das Diarylheptanoid Katsumadain A aus dem Samen der Pflanze *Alpinia katsumadai* aus der Familie der Ingwergewächse isoliert (Grienke *et al.*, 2010). In einer folgenden Studie diente die Struktur von Katsumadain A als Ausgangspunkt für ein virtuelles Screening weiterer NAI (Kirchmair *et al.*, 2011). Dabei zeigte das zweifach isoprenylierte Flavon Artocarpin ein hohes inhibitorisches Potenzial gegen Influenzaviren. Artocarpin kann aus verschiedenen Pflanzenspezies der Gattung *Artocarpus* (Brotfruchtbäume) aus der Familie der Maulbeergewächse (*Moraceae*) extrahiert werden. Bei einem kombinierten Ansatz aus Computermodellierungen und Ethnopharmakologie lassen sich folglich viele neue NAI identifizieren, die bei struktureller Ähnlichkeit zu den Vorläufermolekülen eine verbesserte Wirkung aufweisen.

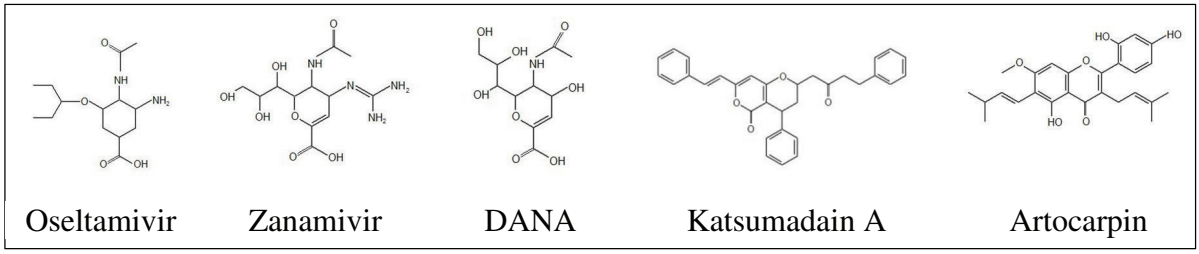

Abbildung 2: Strukturformeln von Neuraminidase-Inhibitoren

1.6 Zielstellung der Arbeit

Das Ziel der vorliegenden Dissertation lag darin, einen therapeutischen Ansatz zu entwickeln, mit dem der letale Synergismus von Influenza-A-Viren und *S. pneumoniae* verhindert werden kann. Im Fokus standen dabei die NA, welche bei beiden Mikroorganismen vorkommen und zur Virulenz beitragen.

Als Ausgangspunkt für alle folgenden Arbeiten sollte zu Beginn geprüft werden, inwieweit die bakteriellen NA ein therapeutisches Target darstellen. Dazu erfolgte zum einen der Nachweis der NA-Gene (NanA, NanB, NanC) in klinischen Isolaten mittels PCR und zum anderen Untersuchungen zur Variabilität der Gene (NanA) durch Sequenzierung und phylogenetische Analysen. Weiterhin galt es die NA-Aktivität der Präzipitate der klinischen Isolate sowie rekombinant hergestellter Enzyme zu bestimmen.

Einen Schwerpunkt dieser Arbeit bildete die Etablierung von Testsystemen zur Prüfung der Aktivität von NAI auf das Bakterienwachstum, die Biofilmbildung und das Adhärenzverhalten. Zur Testvalidierung sollten NAI wie Oseltamivir, Zanamivir und DANA (N-Acetyl-2,3-Dehydro-2-Deoxyneuraminsäure), deren Wirkung gegen Pneumokokken-NA bereits beschrieben wurde, sowie Antibiotika als Kontrollsubstanzen eingesetzt werden. Ein weiterer Schwerpunkt bestand darin, die Aktivität neuer NAI (Katsumadain A, Artocarpin, Azo-Verbindungen) auf die virale und/oder bakterielle NA sowie deren Einfluss auf die Virusreplikation, das Pneumokokken-Wachstum und die Biofilmbildung festzustellen.

Im Schlussteil der Arbeit sollte die Bedeutung von NanA und NanB für die Virusreplikation mit Hilfe eines Koinkubationsmodells untersucht werden. Dazu wurden Lungenepithelzellen (A549-Zellen) mit dem A(H1N1)pdm09-Isolat A/Jena/8178/09 (Jena/8178) infiziert und anschließend mit rekombinanter NanA oder NanB behandelt, um (a) den Einfluss der bakteriellen NA auf die Virusausbreitung sowie den Virusertrag und (b) die Wirkung von NAI auf die Influenzavirusvermehrung in Ab- und Anwesenheit von den bakteriellen NA analysieren zu können.

2 Publikationen

2.1 Publikation 1

Titel: Complementary assays helping to overcome challenges for identifying neuraminidase inhibitors

Zeitschrift: Future Virology

Autoren: Martina Richter*, Lilia Schumann, Elisabeth Walther, Anja Hoffmann, Heike Braun, Ulrike Grienke, Judith M. Rollinger, Susanne von Grafenstein, Klaus R. Liedl, Johannes Kirchmair, Peter Wutzler, Andreas Sauerbrei, Michaela Schmidtke
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Status: publiziert im Februar 2015

Zusammenfassung:

Die Vor- und Nachteile von enzymbasierten Testsystemen zur Identifizierung von neuen NAI standen im Fokus dieser Publikation. Eine Voraussetzung dafür war die Herstellung von Proteinpräzipitat aus Pneumokokken-Kulturen und der Nachweis der NA-Aktivität in diesem Präzipitat. Letzteres wurde eingesetzt, um die Wirkung von NAI und Nicht-NAI im Vergleich zu anderen bakteriellen NA (*Vibrio cholerae*, *Clostridium (C.) perfringens*) und pandemischen Viren in zwei kommerziell verfügbaren Tests (FL- und CL-Test) und in einem neu entwickelten Assay (Hämagglutination-Hemmtest (HA-HT)) zu vergleichen.

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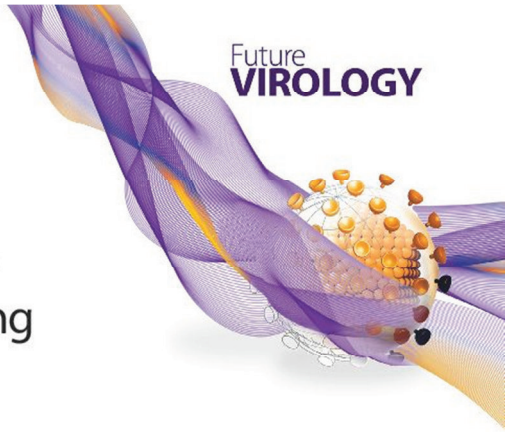

Complementary assays helping to overcome challenges for identifying neuraminidase inhibitors

Martina Richter[1], Lilia Schumann[1], Elisabeth Walther[1], Anja Hoffmann[1], Heike Braun[1], Ulrike Grienke[2,3], Judith M Rollinger[3], Susanne von Grafenstein[4], Klaus R Liedl[4], Johannes Kirchmair[5], Peter Wutzler[1], Andreas Sauerbrei[1] & Michaela Schmidtke*[,1]

ABSTRACT **Aims:** In this study, we analyze the challenges involved in detecting novel neuraminidase inhibitors (NAIs) and offer strategies to overcome them with complementary bioassays. **Materials and Methods:** We investigated the inhibitory activities of NAIs (oseltamivir, zanamivir, DANA, katsumadain A and remazol) as well as non-NAIs (amantadine, nucleozin and rifampicin) on influenzaviral and bacterial (*Streptococcus pneumoniae*, *Clostridium perfringens* and *Vibrio cholerae*) neuraminidases (NAs) with chemiluminescence (CL)- and fluorescence (FL)-based assays. Furthermore, hemagglutination-based NA inhibition assays were established. **Results:** Our study shows three types of signal interference affecting the readout of biochemical assays: self-FL (katsumadain A and remazol), FL quenching (rifampicin) and CL quenching (rifampicin, remazol, nucleozin and katsumadain A). These challenges were overcome by hemagglutination-based assays. **Conclusion:** The latter allow a robust performance in discriminating NAIs and non-NAIs.

KEYWORDS
• assay interference
• *Clostridium perfringens*
• influenza virus
• neuraminidase inhibition assay • neuraminidase inhibitors • self-fluorescence
• signal quenching
• *Streptococcus pneumoniae*
• *Vibrio cholerae*

Influenza A viruses express two surface glycoproteins, hemagglutinin and neuraminidase (NA), which function in a highly balanced manner [1]. The binding of viral hemagglutinin to sialic acid (functioning as receptor on the cell surface) mediates the attachment of the virus to host cells, while influenza NA allows progeny virus particles to elute from infected cells by enzymatic cleavage of terminal sialic acid residues [2]. In this way, viral NA prevents self-aggregation of progeny virions and facilitates their spread across the respiratory tract. NA therefore represents a target of influenza therapeutics.

Because of the widespread resistance of influenza viruses to ion channel blockers, current antiviral therapy options are limited to NA inhibitors (NAIs). These include oseltamivir and zanamivir, both of which are licensed worldwide since 1999, and peramivir and laninamivir, which have been approved in Japan, China and South Korea in 2010 [3,4]. Resistance to NAIs is observed relatively rarely because of the essential catalytic function of these proteins. The emergence and global spread of oseltamivir-resistant H1N1 influenza viruses with H275Y substitution in the season of 2007/2008 [5,6] however

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demonstrated that resistant strains could emerge suddenly. Therefore, the search for novel NAIs is ongoing. Encouraged by the observation that oseltamivir-resistant strains maintain susceptibility to zanamivir, several research groups are investigating structural modifications of oseltamivir [7,8] to overcome loss of activity induced by mutation [4], in particular H275Y. Other groups are searching for novel scaffolds for NAI acting against oseltamivir- and zanamivir-resistant influenza viruses [3,9].

Also bacteria, such as *Streptococcus pneumoniae*, one of the most common causative agent of flu-associated secondary bacterial pneumonia [10], express three different NAs as virulence factors [11,12]. Pneumococcal NAs were shown to play an important role in the lethal synergism of influenza viruses and pneumococci [13]. According to x-ray data, the conformation of the cleavage site of pneumococcal NA resembles that of the viral enzyme [14]. This renders bacterial NA a pharmaceutically relevant target.

Assays most commonly used to monitor the NAI susceptibility of influenza A viruses are based on the detection of chemiluminescence (CL) or fluorescence (FL) signals [4,15–18]. The CL-based NA-inhibition assay (henceforth referred to as CL assay) uses NA-Star® (1,2-dioxetane derivative of sialic acid; Applied Biosystems, Darmstadt, Germany) as chemiluminescent substrate. The FL-based NA inhibition assay (henceforth referred to as FL assay) is based on 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) as fluorogenic substrate. Both assays are standardized and available in kit format. In addition, different protocols for in house FL assay exist. The CL assay is more sensitive than its FL counterpart [4,19]. Studies on the specificity of these assays revealed good correlation between the 50% inhibitory concentration (IC_{50}) values of oseltamivir and zanamivir for NAI susceptible influenza viruses [4,18,19]. However, the FL assay showed the largest difference between IC_{50} values measured for the H1N1 wild-type and the oseltamivir-resistant virus variants with H275Y substitution [4], suggesting that this assay is more sensitive in detecting these drug-resistant viruses. Opposing trends with regard to assay sensitivity were identified for the zanamivir-resistant H3N2 influenza viruses carrying the D151G substitution [20]. These findings imply that resistance-conferring substitutions of amino acids do have an impact on the sensitivity of both assays. According to the manufacturer of the CL and FL assays, both tests are also suitable for the assessment of novel potential inhibitors of viral and bacterial NA. Using these assays, several novel NAIs (synthetic molecules and natural products) have been identified and characterized [7–9,21–23]. Recently, the reliability of these assays was questioned, specifically for the testing of flavonoids, the most prominent scaffolds found among various chemical classes of NA inhibiting natural products [24]. Some of them were reported as quenching the FL signal of the fluorogenic cleavage product of MUNANA, 4-methylumbelliferone (4-MU), leading to false-positive results [25]. For these flavonoids also quenching of the CL signal was reported [24]. It was suggested that the quenching effects measured at flavonoid concentrations of 142 and/or 714 μM were mediated by the phenyl-benzpyran scaffold.

Miniaturized assays using fetuin as substrate provide a further possibility to measure anti-NA activity. These assays are typically used to determine the titer of anti-NA antibodies [26,27]. They were also evaluated for their suitability to detect NAI resistance [28]. Fetuin-based assays are colorimetric assays. In contrast to antibodies, oseltamivir and zanamivir, many natural compounds are colored and hence hold the risk of color-based assay interference.

In searching for novel inhibitors of viral and bacterial NAs, we tested more than 500 small molecules for their inhibitory activity on various NAs. This selection comprised synthetic molecules from virtual screening and natural products selected by empirical knowledge (data not shown). As a result, we identified several compounds showing strong inhibitory activity in the CL assay but poor or no measurable activity in the FL assay. In this work, we present a representative collection of compounds that we investigated in detail regarding their behavior in these biochemical assays: known NAIs (oseltamivir, zanamivir and *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid [DANA]); novel NAIs (katsumadain A [21], remazol [9]); and non-NAIs (rifampicin, nucleozin, amantadine). Our aim was to evaluate the significance of the assays for a diverse set of compounds and rationalize the underlying biochemical processes. We also elaborated alternative assays working under conditions similar to the physiological environment (pH, substrate and salt concentration) that reveal more reliable results. In particular, we focused on measuring the inhibition of viral NA

activity in elution of hemagglutination (HA); and of bacterial NAs lectin-based HA (both henceforth referred to as HA assays).

Material & methods

• Compounds

Oseltamivir carboxylate (GS4071) and zanamivir (GG167) were obtained from GlaxoSmithKline (Uxbridge, UK) and F. Hoffmann-La Roche AG (Basel, Switzerland), respectively. *N*-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA), rifampicin, nucleozin, amantadine hydrochloride and remazol Brilliant Blue R (remazol) were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany).

Katsumadain A, previously isolated from the seeds of *Alpinia katsumadai* Hayata [21], and nucleozin were dissolved in dimethyl sulphoxide (DMSO). The diluent of all other compounds was Aqua bidest. Compound stocks (10 mM) were stored at 4°C, with the exception of DANA and rifampicin, which were stored at -20°C, until use.

• Human erythrocytes

Human erythrocytes were obtained from the Institute of Transfusion Medicine, University Hospital Jena, Jena, Germany. The erythrocytes were collected in additive solution (SAG-M) and stored at 4°C. Before use, blood cells were washed three-times in phosphate-buffered saline (PBS) without Ca^{2+} and without Mg^{2+} (900× g, 4°C, 8 min).

• Cell line & virus propagation

Madin-Darby canine kidney cells (Friedrich-Loeffler Institute, Riems, Germany) were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin serum and 2 mM L-glutamine.

Stock of pandemic H1N1 influenza virus A/Jena/5258/09 (influenza A virus [22]) was prepared in serum-free Eagle's minimum essential medium containing 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 2 µg/ml trypsin and 0.1% sodium bicarbonate in mycoplasma-free Madin-Darby canine kidney cells. Aliquots were stored at -80°C until use.

• Preparation of precipitated pneumococcal total proteins

S. pneumoniae strain DSM20566 was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). To get a stock of *S. pneumoniae* strain DSM20566, bacterial cultures were grown on Columbia blood agar plates at 37°C in 5% CO_2 overnight. Aliquots were frozen using a cryovial system (CRYOBANK™; Mast Diagnostika, Reinfeld, Germany).

For the experiments, frozen bacteria cells were plated on Columbia agar containing 5% Sheep Blood (Becton Dickinson GmbH, Heidelberg, Germany) in agar Petri dishes and cultured at 37°C in 5% CO_2 overnight. Thereafter, pneumococci were inoculated into flasks containing 100 ml of heart brain infusion (HBI) broth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). After incubation at 37°C overnight with shaking, bacteria were diluted 1:10 in ice-cold absolute ethanol and kept at -20°C overnight. Afterward, the tubes were centrifuged at 3000× g for 20 min at 4°C before the supernatant was removed. The precipitate was washed with ice-cold 70% ethanol. Subsequently to centrifugation (3000× g; 10 min; 4°C), the ethanol supernatant was removed. After drying, ethanol-precipitated proteins were dissolved in morpholineethanesulfonic acid (MES) buffer (32.5 mM MES; 4 mM calcium chloride, pH 6.5) for CL and FL assays or in PBS for HA assays. Aliquots were stored at -20°C until use.

• Purified bacterial NA

Lyophilized NA of *Clostridium perfringens* (NanJ) was purchased (Sigma-Aldrich GmbH), dissolved in PBS and stored at -20°C until use. NA from *Vibrio cholerae* was ordered in a buffered aqueous solution (Sigma-Aldrich GmbH) and stored at 4°C.

• CL assay

NA activity was determined in 96-well white U-bottom microplates (Greiner bio-one GmbH, Frickenhausen, Germany) using the CL-based NA-Star assay (NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit, Applied Biosystems, Darmstadt, Germany), according to the manufacturer's instructions with slight modifications.

Briefly, 25 µl test compounds in ten half-log serial dilutions in NA-Star assay buffer (26 mM MES; 4 mM calcium chloride; pH 6.0) were mixed with 25 µl of a certain bacterial NA protein or virus dilution (signal-to-noise ratio: ~40:1) and incubated at 37°C for 20 min. After adding 5 µl of 500× diluted NA-Star substrate,

the plates were incubated at room temperature for 30 min. Reaction was stopped by adding 55 µl of NA-Star accelerator. CL was determined by using the microplate reader FLUOstar Omega (BMG Labtech GmbH, Ortenberg, Germany). At least three individual experiments were performed for the calculation of the 50% inhibitory concentration of NA with the JASPR curve-fitting software [6].

• FL assay

The FL assay was run on 96-well black flat-bottom microplates (Greiner bio-one GmbH), as published previously [29] with some modifications.

Briefly, serial dilutions of virus stocks or bacterial protein solutions in MES buffer (32.5 mM MES; 4 mM calcium chloride; pH 6.5) were incubated with the substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate (MUNANA 100 µM; Sigma-Aldrich GmbH) at 37°C for 2 h. After analysis of the FL signal, the NA dilution that resulted in a good measurable FL signal in the linear range of 4-MU was selected for the inhibition test.

For the FL assay, 10 µl compounds in seven tenfold serial dilutions in FL-MES buffer were mixed with 10 µl of the predetermined bacterial NA protein or virus dilution and incubated at 37°C for 20 min. After adding 30 µl of MUNANA (100 µM) and further incubation at 37°C for 2 h, reaction was terminated by adding 150 µl stop solution (0.1 M glycine, 25% absolute ethanol, pH 10.7). FL was read (excitation wavelength of 355 nm and emission wavelength of 460 nm) using the microplate reader FLUOstar Omega (BMG Labtech GmbH). At least three independent experiments were performed for the calculation of the IC_{50} values with the JASPR curve fitting software [6].

• Studies on the interference of small molecules with the CL & FL assays

Compound solutions of 100, 10 and 1 µM in MES buffer were analyzed for self-FL, self-CL and quenching of the assay signals (minimum of three independent assays).

To determine self-FL and self-CL, MES buffer without (negative control) and with compounds were incubated under the respective assay conditions. After addition of stop solution (FL assay) or NA-Star accelerator (CL assay), FL or CL was read as described above. The percentage of self-FL or self-CL was calculated by setting the values of MES buffer to 100% (background).

For determination of the reduction of FL signal, 30 µl of the fluorescent 4-MU (the FL of 5.21 µM of 4-MU corresponds to FL values determined after substrate cleavage under assays conditions; Sigma-Aldrich GmbH) was added to buffer without and with compounds. Negative controls contained MES buffer alone. After incubation for 2 h at 37°C and addition of 150 µl of stop solution, the FL signal was read as described above. The percentage of reduction in FL signal was calculated by setting the blank-corrected FL of 4-MU to 100%.

For determination of CL signal reduction, a virus dilution (A/Jena/5258/2009) was incubated with MES buffer and 100 µM NA-Star substrate as described above. After addition of NA-Star accelerator containing different compound concentrations, the percentage of CL signal reduction was calculated by setting the blank-corrected CL of the virus control with NA-Star accelerator to 100%.

• HA-based functional NA inhibition assays

To test the NA inhibitory activity of compounds under physiological conditions (neutral pH, low salt concentration and natural substrate), 25 µl of influenza A virus dilution resulting in HA (twofold of the highest virus dilution that caused an agglutination of the erythrocytes) was added to an equal volume of serial half-logarithmic compound dilutions in PBS (maximum end concentration: 100 µM) or PBS (HA control) in a V-bottom 96-well polystyrene microplate. Compound dilutions without virus were used as additional control (toxicity and agglutination control). After incubation at 37°C under 5% CO_2 atmosphere for 20 min, 50 µl of a 0.75% human erythrocytes solution was added for 2 h at 4°C to allow HA. After further incubation at 37°C for 1 h (activation of NA), the plate was inclined and scanned to monitor elution of HA by the activated NA as well as the inhibition of this reaction by test compounds. The compound concentration that inhibits HA elution was defined as the minimum NA inhibitory concentration **(Figure 1A)**. Each compound was tested at least three times.

To evaluate bacterial NA activity as well as its inhibition by NAI, the previously published lectin-based HA assay [30] was adapted. In a pretest, NA activity was measured with 5 µl of a 25% human erythrocyte suspension (in PBS buffer) that was subjected to 100 µl of serially diluted (dilution factor 2) purified

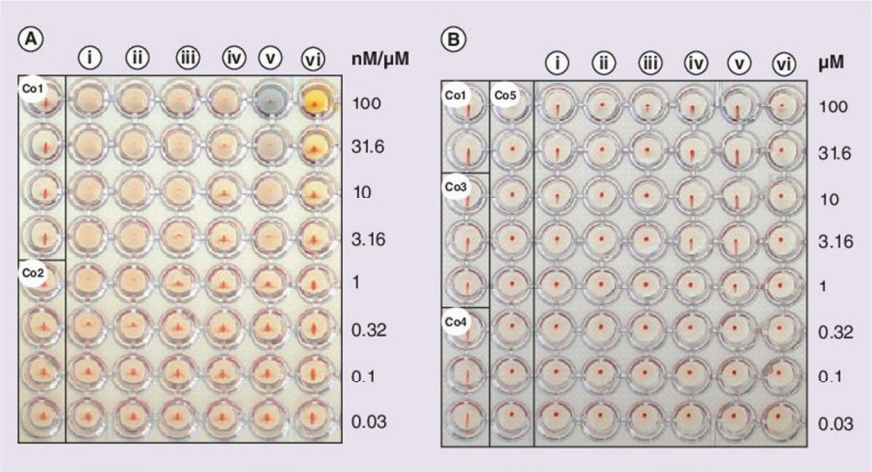

Figure 1. Representative examples of photographs of hemagglutination-based neuraminidase inhibition assays. (A) Influenza virus A/Jena/5258/2009 and **(B)** precipitated total bacterial protein of pneumococci strain DSM20566 were treated with serial half-logarithmic dilutions of **(i)** oseltamivir, **(ii)** zanamivir, **(iii)** N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA), **(iv)** katsumadain A, **(v)** remazol and **(vi)** rifampicin from 100 to 0.03 µM (exception: **[i & ii]** in **[A]** ranged from 100 to 0.03 nM). Controls not showing hemagglutination are human erythrocytes (Co1) in phosphate-buffered saline, (Co2) with viral neuraminidase, (Co3) with lectin (50 µg/ml) and (Co4) with precipitated bacterial protein (1:2000-diluted). Human erythrocytes with peanut lectin (50 µg/ml) as well as precipitated bacterial protein (1:2000-diluted) served as positive control (Co5).

NA solutions of *C. perfringens* and *V. cholerae* or precipitated pneumococcal total protein in flat-bottom 96-well polystyrene microplates (Greiner bio-one GmbH). After incubation at 37°C in 5% CO_2 for 4 h, 20 µl of this protein-erythrocytes suspension was added to 20 µl of *Arachis hypogaea* lectin (Sigma-Aldrich GmbH; 50 µg/ml) in V-shaped 96-well polystyrene microplates (Greiner bio-one GmbH) and incubated at 4°C overnight. Thereafter, the plates were inclined and the lowest concentration of purified NA solutions or precipitated pneumococcal total protein causing HA was determined (mean value of three independent assays) and used for NA inhibition assay. To analyze NA inhibition by test compounds, 50 µl of double-concentrated predetermined bacterial protein dilution was added to 50 µl of double-concentrated serial half-logarithmic compound dilutions (maximum concentration: 100 µM) or PBS (NA activity control) in a flat-bottom 96-well polystyrene microplate. After incubation at 37°C and 5% CO_2 for 20 min, allowing compound binding to NA, 5 µl of 25% erythrocytes solution was added. Following careful homogenization and incubation under the same conditions for 4 h, NA activity was determined as described above via HA. Negative controls included erythrocytes in PBS, a mixture of erythrocytes and bacterial proteins, and a mixture of erythrocytes and lectin. The compound concentration that abrogated HA was defined as minimal NA inhibitory concentration. An example is shown in **Figure 1B**. Each compound was tested at least three-times. In addition, a cocktail of erythrocytes, lectin and compounds without bacterial protein (one well per compound concentration) as well as erythrocytes and compounds alone was included to confirm that HA was not caused by the interaction between compounds and erythrocytes.

• Statistical analysis

Mean and standard deviation (SD) values were analyzed using Microsoft Excel 2010. In the studies on the interference of small molecules with the CL and FL assay, the cutoff value was set at mean ± 2 SD of control (without compound). Statistically, significant differences were analyzed by using Welch's *t*-test (Microsoft Excel) after confirming the normal distribution of data.

Results

• NA inhibitory activity determined with CL & FL assays

In accordance with previous studies [4,15,16,28,31,32], oseltamivir and zanamivir inhibited the viral NA at concentrations ≤1 nM in the CL and FL assays (Table 1). In addition, oseltamivir showed moderate, poor or no activity for *S. pneumoniae*, *C. perfringens* and *V. cholerae* NA, respectively. Zanamivir exerted poor activity against *V. cholerae* NA and was inactive against *S. pneumoniae* and *C. perfringens* NA (up to the maximum tested concentration of 100 μM). In contrast, the less virus-specific NAI DANA inhibited viral as well as bacterial NA at concentrations <10 μM.

Like DANA, the two recently described novel NAIs katsumadain A and remazol [9,21] exerted good inhibitory activity against all studied NAs (Table 1). Their IC_{50} values determined with the CL assay were similar (for viral NA) or lower than that of DANA (for bacterial NA). The IC_{50} values of katsumadain A and in part also of remazol measured in the FL assay did not show a consistent trend.

Intriguingly, the non-NAI rifampicin exhibited strong inhibitory activity in the CL assay, with IC_{50} values between 0.03 and 0.11 μM, whereas weak or no activity was measured in the FL assay (Table 1). Similar observations were made for nucleozin, with IC_{50} values between 1.95 and 9.11 μM in CL assay and no activity in FL assay (Table 1). In contrast to that, amantadine did not show activity in both assays with bacterial NA (Table 1). Weak activity was observed against viral NA in both assays.

With the exception of pH1N1 NA and zanamivir, any of the measured IC_{50} values of both NAIs and non-NAIs were lower for the CL assay when compared with the ones reported by the FL assay (Table 1). The ratio between IC_{50} values of approved and novel NAIs measured with the FL and CL assays were in the range of 1–2 and 2–74, respectively. Differences were particularly high for katsumadain A and remazol. For the antibiotic rifampicin, this ratio was extreme (212 to >3750). These discrepancies nourished doubts about the reliability of the biochemical assays for testing of novel potential NAIs.

• Interference with FL & CL signals

Recently, it was shown that quenching of FL and CL signals can lead to false-positive results [24,25]. Therefore, we analyzed the capacity of compounds to reduce the FL and/or CL signal. In addition, self-FL and self-CL of all investigated compounds were analyzed at assay conditions as such sample properties may induce false-negative results.

As shown in Figure 2A, katsumadain A exhibited a high level of self-FL at concentrations similar to or greater than 10 μM when compared with the background fluorescence of the assay buffer under standard assay conditions. Furthermore, remazol exhibited fluorescence at 100 μM concentration (Figure 2A). There was no self-CL detected for any of the studied compounds (results not shown).

Rifampicin showed strong signal quenching in both assay formats (Figure 2B & C). Remazol, nucleozin and katsumadain A reduced also the CL signal (Figure 2C). These results corroborated our concerns regarding the reliability of these assays.

• NA inhibition as determined by HA assays

In search for functional assays that do not suffer from these types of interference, we investigated ways to quantify the inhibitory activity of small molecules *via* assays that measure the biological function of viral and bacterial NAs during the process of pathogen–erythrocyte interaction.

Binding of influenza viruses to erythrocytes induces HA at 4°C. At this temperature, viral NA is inactive. It becomes activated and can elute erythrocytes at 37°C, provided there is an adequate balance of hemagglutinin-NA functionality, as it is the case for the pH1N1 used in the present study. We assumed that NAIs would inhibit this reaction at low micromolar concentrations. This assay showed NA inhibition for oseltamivir, zanamivir, DANA and remazol (Table 1). Katsumadain A induced HA of erythrocytes at concentration of 31.6 and 100 μM by themselves and was inactive at lower concentrations. The non-NAIs nucleozin and amantadine showed a very weak effect at the highest testing concentration. No measurable effect was found for rifampicin.

In contrast to viruses, bacteria do not hemagglutinate erythrocytes by themselves. However, previously it was shown that after removal of sialic acids by bacterial NA, peanut lectin can induce the HA of erythrocytes [30]. The block

of bacterial NAs should prevent this process. Using this experimental approach, oseltamivir significantly inhibited pneumococcal NA at about 2.1 µM. Oseltamivir however did not exert any activity against *C. perfringens* and *V. cholerae* NA. In the presence of about 54 µM of zanamivir, the activity of *V. cholerae* NA was inhibited. The bacterial NAs of *S. pneumoniae* and *C. perfringens* were not inhibited. DANA reduced the activity of all of the bacterial NAs by 50% at concentrations between 17.2 and 45.3 µM. The best inhibitory activity against bacterial NAs was exerted by katsumadain A and remazol. No activity was found for compounds of the negative control, rifampicin, nucleozin and amantadine.

Discussion

In this work, we exemplify the scope and limitations of functional NA inhibition assays by showing the inhibitory activity of three well-known NAIs (oseltamivir, zanamivir and DANA), two novel NAIs (katsumadain A and remazol) and three non-NAIs (rifampicin, nucleozin and amantadine) against one influenza virus and three bacterial NAs. Self-FL and quenching of FL and CL signals were identified as potential issues that can lead to false-negative or false-positive results in the FL- and CL-based biochemical assays. These problems were overcome by establishing HA assays.

Known NAIs showed significant inhibition of viral NA. They were much weaker inhibitors of bacterial NAs (if showing any activity at all). In contrast to that, DANA inhibited a broad spectrum of NAs at moderate levels of activity. These findings correspond well to earlier results for bacterial NAs [14,33].

Furthermore, with the exception of the activity measured for zanamivir on pH1N1, IC_{50} values obtained with the established NAIs for viral NA correspond to the previously described higher susceptibility of the CL assay in comparison to FL assay [4]. This difference in assay susceptibility was also observed for *S. pneumoniae*, *C. perfringens* and *V. cholerae* NA with oseltamivir and DANA in the present study. In a similar fashion, the IC_{50} values of katsumadain A and remazol determined with the CL assay were markedly lower than the ones observed with the FL assay for viral and bacterial NA, whereby earlier reported IC_{50} values for influenza viruses could be confirmed [9,21]. Intriguingly, even the

Table 1. Mean 50% inhibitory concentration values (chemiluminescence and fluorescence assays) and minimal inhibitory concentration (hemagglutination assay) with standard deviation (µM) against viral and bacterial neuraminidases.

Compound	Influenza A virus NA			*Streptococcus pneumoniae* NA			*Clostridium perfringens* NA			*Vibrio cholerae* NA		
	CL	FL	HA	CL	FL	HA	CL	FL	HA	CL	FL	HA
Oseltamivir	0.00022 ± 0.00005	0.00048 ± 0.00024	0.0007 ± 0.0004	0.65 ± 0.13	1.12 ± 0.32	2.08 ± 1.25	43.48 ± 16.51	61.34 ± 19.21	100.00 ± 0.00	Not active	Not active	Not active
Zanamivir	0.00032 ± 0.00011	0.00014 ± 0.00006	0.0014 ± 0.0012	Not active	Not active	Not active	Not active	Not active	Not active	20.56 ± 3.27	42.46 ± 15.77	54.40 ± 39.49
DANA	0.59 ± 0.26	1.86 ± 0.37	4.87 ± 3.42	2.59 ± 0.81	9.95 ± 1.38	45.28 ± 30.59	1.59 ± 0.29	4.94 ± 1.08	2440 ± 12.47	2.49 ± 0.51	6.06 ± 0.22	17.20 ± 12.47
Katsumadain A	0.43 ± 0.15	48.36 ± 13.87	Not evaluable[†]	0.94 ± 0.49	13.35 ± 4.63	3.16 ± 0.00	0.10 ± 0.04	2.81 ± 2.19	2.44 ± 1.25	0.39 ± 0.10	14.99 ± 4.66	1.72 ± 1.25
Remazol	0.26 ± 0.05	2.04 ± 1.31	2.62 ± 1.03[‡]	0.19 ± 0.11	0.32 ± 0.12	0.66 ± 0.39	0.03 ± 0.01	0.36 ± 0.15	7.72 ± 3.95	0.47 ± 0.24	35.04 ± 18.35	3.16 ± 0.00
Rifampicin	0.03 ± 0.02	85.49 ± 21.61	Not active	0.11 ± 0.06	22.41 ± 5.44	Not active	0.03 ± 0.01	Not active	Not active	0.03 ± 0.01	Not active	Not active
Nucleozin	1.95 ± 0.68	Not active	100.00 ± 0.00	9.11 ± 4.91	Not active	Not active	2.52 ± 0.71	Not active	Not active	3.24 ± 0.05	Not active	Not active
Amantadine	11.52 ± 2.28	37.59 ± 2.35	65.80 ± 39.49	Not active	Not active	Not active	81.99 ± 39.86	Not active	Not active	>100	Not active	Not active

Mean 50% inhibitory concentration and standard deviation for CL and FL assays and minimal inhibitory concentration in HA assay against viral NA of a pardemic H1N1 influenza A virus and three bacterial NA determined in at least three independent assays. The maximum tested concentration was 100 µM.
[†]HA of erythrocytes by the test compound up to about 31.6 µM.
[‡]Inhibition of virus-induced HA of erythrocytes by the test compound up to about 31.6 µM.
CL: Chemiluminescence; DANA: N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid; FL: Fluorescence; HA: Hemagglutination; NA: Neuraminidase.

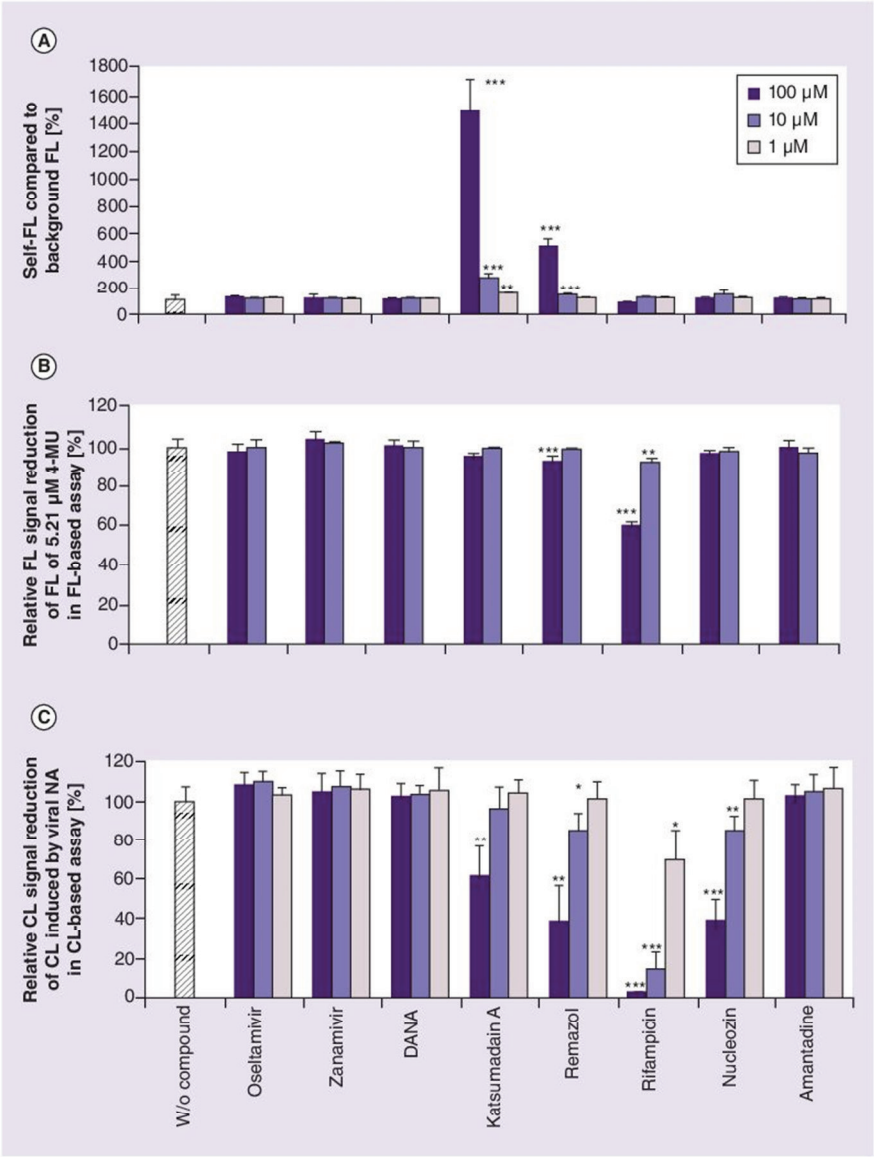

Figure 2. Self-fluorescence and quenching of fluorescence and chemiluminescence signal by test compounds. Bars represent the mean percentage of self-FL **(A)**, FL signal reduction **(B)** and CL signal reduction **(C)** with standard deviation from at least three independent assays.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
CL: Chemiluminescence; DANA: N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid; FL: Fluorescence; NA: Neuraminidase; W/o: Without.

non-NAIs included as control confirmed this tendency.

The results from control studies show that the observed difference in sensitivity between the CL and FL assays is not necessarily correct. It may result from self-FL of the tested compound as observed for katsumadain A. The self-FL of katsumadain A at 100 and even at 10 µM can

lead to a deterioration of the inhibitory effect in cases where the IC_{50} against a specific NA is greater than 10 µM, as observed for viral, *S. pneumoniae* and *V. cholerae* NA. In contrast, IC_{50} values of katsumadain A of about 1 µM, as determined for *C. perfringens* NA, are unlikely hampered by self-FL. The moderate self-FL of remazol at 100 µM may be neglected for viral, pneumococcal and *C. perfringens* NA because its IC_{50} values are significantly below this threshold. However, an impact on the activity of remazol against *V. cholerae* NA cannot be excluded. Self-CL of tested compounds was not detected up to the maximum tested concentration but cannot be ruled out in general.

Results of additional control studies indicate that an overestimation of compound activity in FL as well as CL assay may happen, if a compound quenches the light signal used for detection. This is not the case for established NAIs like oseltamivir and zanamivir. Thus, these assays have their application area in characterizing resistance profiles for these compound classes [4]. Bioactive molecules often contain aromatic moieties, and these are prone to causing interference with light signals, for example rifampicin quenches both the FL and CL signals. In contrast to quenching effects recently observed with flavonoids at high concentrations of 132 and 714 µM [24,25], rifampicin quenched the CL signal at concentrations in the low micromolar range, in some cases even in concentrations <1 µM. Thus, low and consistent IC_{50} values (0.03–0.11 µM) were observed for rifampicin in all CL inhibition assays independently from the studied NAs. We assume that the adsorption spectrum of rifampicin at pH 10 overlaps with the CL or FL emission wavelength. Thus, the presence of rifampicin feigns that the enzymatic product is not present, which is erroneously interpretive as enzyme inhibition.

According to our experience, the 4 mM Ca^{2+} concentration in the assay buffer that stabilizes the enzyme activity [34] can represent a further issue for the testing of potential inhibitors in both FL and CL assays as it may cause a decrease of their solubility [Hoffmann A, Unpublished Data].

The HA assays, adapted for the search of novel NAIs in the present study, have several advantages. They are running at conditions more similar to the physiological environment, at neutral pH and with a natural substrate (cell-surface-sialidated glycoconjugates), but most importantly, their readout is highly reliable and robust. Compared with the biochemical assays, they are however not available as commercial kits, hence requiring a high level of expertise. Moreover, before using HA-based assays with an influenza virus, an adequate balance between hemagglutinin and NA of the specific virus needs to be confirmed, in analogy to cytopathic effect inhibition or plaque reduction assays [4,15,35]. According to our experience, the HA assays with human red blood cells and the selected pH1N1 are highly reproducible. We could not observe an impact of different erythrocyte preparations on the results. In contrast, the host species are highly important. Because of differences in

Table 2. Advantages and disadvantages of chemiluminescence, fluorescence and hemagglutination assays.

Assay type	Advantages	Disadvantages
CL assay	Available as commercial kit Highly sensitive Suitable for high-throughput screening Highly suitable for testing sugar-like molecules	May generally overestimate biological activity of novel compounds Strong interference possible via signal quenching Aggregation effects partially due to 4 mM Ca^{2+} Insufficient for confirming inhibitory activity of small molecules without complementary assay Very expensive
FL assay	Available as commercial kit Suitable for high-throughput screening Enzyme kinetics studies possible Highly suitable for sugar-like molecules	Strong interference possible via signal quenching or self-FL Aggregation effects partially due to 4 mM Ca^{2+} Insufficient for confirming inhibitory activity of small molecules without complementary assay Expensive
HA assay	Highly robust assay readout Medium throughput system Use as primary assay for identifying potential NAI Neutral pH, low salt concentration, natural substrate	High demands in expertise Dependent on hemagglutinin-neuraminidase balance Not available as commercial kit

CL: Chemiluminescence; FL: Fluorescence; HA: Hemagglutination; NAI: Neuraminidase inhibitor.

sialic acid expression in different hosts, they are directly related to the hemagglutinin-NA functions balance (sialic acid recognition and cleavage, respectively). Establishing the assay, we selected an H1N1 influenza virus with good hemagglutinin binding capacity to human red blood cells resulting in a high HA titer at 4°C; and with a NA that was capable to elute these cells at 37°C. Given a suitable balance between the viral proteins, the HA assays were able to reliably detect any known and tested NAIs and did not report any non-NAIs as active. Due to the induction of erythrocyte HA at concentrations >31.6 µM, no activity value could be determined for katsumadain A on viral NA. Nucleozin and amantadine exhibited poor inhibitory activity when tested at high concentrations on viral NA in the HA assay. We suggest that both small compounds can inhibit the viral enzyme in high concentrations in a more or less (un)specific manner.

In conclusion, the compared assays differ in their applicability, and each of them comes with individual advantages and disadvantages (Table 2). Our findings warrant the use of HA assays in the search for novel potential NAIs, in particular when seeking to identify agents with dual inhibitory activity against the viral and bacterial proteins.

Conclusion

The results of this study demonstrate that the established HA assays are particularly valuable as they allow the analysis of viral and bacterial NA function under more physiological conditions. They help to overcome the light interference issues related to the use of the artificial substrates NA-Star and MUNANA. FL and/or CL assays can be used to confirm the activity found in HA assays and to study the spectrum of activity or binding mechanism in enzyme kinetics. For the reliable use of these assays, evaluation of self-FL/CL and FL/CL signal quenching is essential because interference with light signal needs to be excluded.

Future perspective

There is an urgent need for the development of new, potent anti-influenza drugs. Current biochemical assays are in principle able to identify novel NAIs, but various types of assay interference may affect readout. It is hence important to use complementary, cell-based assay systems for the confirmation of any hits. The concerted use of biochemical and cell-based assays will allow the development of new drugs, which if applied in the form of combination therapy, may help to prevent the development of drug resistance. Further to that, targeting bacterial NA with novel inhibitors appears to a very promising strategy for the prophylaxis and treatment of secondary pneumococcal infections.

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EXECUTIVE SUMMARY

- For bacterial neuraminidases (NAs), the chemiluminescence-based inhibition assay is more sensitive than its fluorescence-based counterpart.
- IC_{50} values of small molecules tested in this study in general strongly depended on the specific NA under investigation.
- Interference of several compounds with fluorescence and/or chemiluminescence signals can lead to an under- or overestimation of inhibitory activity.
- Hemagglutination (HA)-based NA inhibition assays with human erythrocytes showing the inhibition of elution of hemagglutinated erythrocytes by viral NA or of lectin-induced HA in the presence of bacterial NA represent good alternative assays to identify novel potential inhibitors.
- HA-based NA inhibition assays enable the screening for novel inhibitors under more physiological conditions (pH, salt concentration and substrate). They produce a highly reliable readout.

'Natural Lead Structures Targeting Influenza' (P24587). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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2.2 Publikation 2

Titel: Antipneumococcal activity of neuraminidase inhibiting artocarpin

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Zusammenfassung:

In dieser Veröffentlichung wurde gezeigt, dass die NA (Präzipitat und NanA) von Pneumokokken gegenüber bekannten NAI wie Oseltamivir und DANA, jedoch nicht gegenüber Zanamivir, im niedrigen mikromolaren Bereich empfindlich ist. Der neue antivirale Inhibitor Artocarpin hemmt zusätzlich das bakterielle Wachstum, die Biofilmbildung und die Adhärenz (an Lungenepithelzellen; A549-Zellen) von Pneumokokken. Artocarpin stellt deshalb einen geeigneten Naturstoffkandidaten für weitere Untersuchungen dar.

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Antipneumococcal activity of neuraminidase inhibiting artocarpin

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ABSTRACT

Streptococcus (*S.*) *pneumoniae* is a major cause of secondary bacterial pneumonia during influenza epidemics. Neuraminidase (NA) is a virulence factor of both pneumococci and influenza viruses. Bacterial neuraminidases (NAs) are structurally related to viral NA and susceptible to oseltamivir, an inhibitor designed to target viral NA. This prompted us to evaluate the antipneumococcal potential of two NA inhibiting natural compounds, the diarylheptanoid katsumadain A and the isoprenylated flavone artocarpin. Chemiluminescence, fluorescence-, and hemagglutination-based enzyme assays were applied to determine the inhibitory efficiency (IC_{50} value) of the tested compounds towards pneumococcal NAs. The mechanism of inhibition was studied via enzyme kinetics with recombinant NanA NA. Unlike oseltamivir, which competes with the natural substrate of NA, artocarpin exhibits a mixed-type inhibition with a K_i value of 9.70 μM. Remarkably, artocarpin was the only NA inhibitor (NAI) for which an inhibitory effect on pneumococcal growth (MIC: 0.99–5.75 μM) and biofilm formation (MBIC: 1.15–2.97 μM) was observable. In addition, we discovered that the bactericidal effect of artocarpin can reduce the viability of pneumococci by a factor of >1000, without obvious harm to lung epithelial cells. This renders artocarpin a promising natural product for further investigations.

Introduction

S. pneumoniae is responsible for the majority of pneumonia cases and the death of about 1.2 million young children worldwide each year (18% of all deaths of children under the age of five) (Black et al., 2010; Krzysciak et al., 2013). Spreading of *S. pneumoniae* in the nasopharynx and surrounding tissues causes the clinical manifestation. The diseases range from mild upper respiratory tract infections, such as acute otitis media, sinusitis, and pneumonia, to severe and potentially life-threatening conditions, such as meningitis and sepsis, by bacterial invasion of the bloodstream (Simell et al., 2012). Additionally, a lethal synergism between pulmonary coinfections with influenza virus and *S. pneumoniae* has been established, accounting for the excess mortality during influenza epidemics and pandemics whereat pneumococcal NAs were found to support viral release and spread in the lung (Kash et al., 2011; McCullers and Bartmess, 2003).

Pneumococcal NAs (NanA, B, and C) belong to a wide range of surface-associated proteins interacting with eukaryotic cells, extracellular matrix proteins, and serum proteins (Lofling et al., 2011). They catalyze the removal of terminal sialic acid residue from various glycoconjugates on cell surface (Taylor, 1996), by which means they reveal receptors for bacterial adhesion (King et al., 2006). They also promote upper (Tong et al., 2000) and lower (Orihuela et al., 2004) airway colonization, biofilm formation, and mucosal infection (Brittan et al., 2012; King et al., 2006; Soong et al., 2006). The released sialic acids serve as a carbon source for the bacteria and represent a trigger for biofilm formation (Trappetti et al., 2009). In

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Table 1
The *S. pneumoniae* strains studied with both genetic and phenotypic identification of NanA activity.

Strain	Source	*nanA*	*nanB*	*nanC*	NA activity[a]
DSM20566	Reference strain	×	×	–	×[b]
Recombinant	NanA of DSM20566	×	–	–	×[b]
DSM14378	Reference strain	×	×	–	×
D39	Reference strain	×	×	–	×[b]
CF6937	Cystic fibrosis	×	–	×	×
CF8919	Cystic fibrosis	×	×	–	×
BC7326	Blood culture, sepsis	×	×	×	×
BC57	Blood culture, sepsis	×	×	–	×
PN8828	Pneumonia	×	×	–	×
CJ9400	Conjunctivitis	×	×	–	×[b]

[a] NA activity was proved in the FL- and CL-based assays.
[b] Lectin-based HA assay was additionally performed to demonstrate the NA activity.

addition, pneumococcal NAs contribute critically to inflammation and mortality associated with sepsis (Chen et al., 2011).

The essential roles of NAs during coinfection with influenza viruses and in pathogenesis of pneumococcal strains render them an attractive target for therapeutic intervention (Taylor, 1996). Blocking NA activity with small-molecule inhibitors in the intestinal perforation model of sepsis led to a substantial reduction of the inflammatory response and subsequent morbidity (Chen et al., 2011; Paulson and Kawasaki, 2011). Administration of the influenza virus-specific neuraminidase inhibitor (NAI) oseltamivir interrupted the lethal synergism between influenza virus and *S. pneumoniae* and prevented excess mortality from secondary bacterial pneumonia in a mouse model (McCullers and Bartmess, 2003). Currently, there are only two influenza NAIs (zanamivir and oseltamivir) prescribed worldwide for the treatment and control of influenza (Grienke et al., 2012). Their inhibitory potencies are either weak (zanamivir) or medium (oseltamivir) (Gut et al., 2011) to pneumococcal NA.

Recently, we discovered the diarylheptanoid katsumadain A and the isoprenylated flavone artocarpin as novel NAIs acting against influenza viruses (Grienke et al., 2010; Kirchmair et al., 2011). In the present study, we evaluated the antipneumococcal potential of both natural product NAIs. We analyzed their inhibitory effect on pneumococcal NA and performed enzyme kinetic studies to understand the molecular mechanism of their inhibition of NanA. In addition, we investigated whether these NAIs affect the bacterial growth, adsorption, biofilm formation, and viability.

Material and methods

Compounds

Oseltamivir carboxylate GS4071 (oseltamivir; Roche AG, Basel, Switzerland), zanamivir (GlaxoSmithKline, Brentford, UK), DANA (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid), and rifampicin (both purchased from Sigma-Aldrich, Deisenhofen, Germany) were dissolved in water as 10 mM stock solutions. Rifampicin was stored at −20 °C. Artocarpin (Quality Phytochemicals LLC. East Brunswick. NJ, USA) and katsumadain A, previously isolated from the seeds of *Alpinia katsumadai* Hayata (Grienke et al., 2010), were dissolved in DMSO as 10 mM stock solutions and stored at 4 °C. Their HPLC purity revealed to be >98%.

Bacterial strains, cells, media, and pre-culture conditions

Six *S. pneumoniae* clinical isolates were collected from patients with different symptoms (Table 1). Two reference strains DSM20566 (serotype 1, ATCC 33400) and DSM14378 (serotype 5, ATCC 6305) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Heidelberg, Germany). *S. pneumoniae* D39 (serotype 2) was kindly provided by ZIK Septomics (Jena, Germany).

All nine strains used in this study were grown on Columbia blood agar plates supplemented with 5% sheep blood (Becton Dickinson GmbH. Heidelberg. Germany) at 37 °C in an atmosphere enriched with 5% CO_2 overnight. During pre-cultivation, bacteria were grown in brain heart infusion broth (BHI) with slightly shaking at 37 °C for 4 to 5 h. This incubation time corresponded with the mid-exponential phase of bacterial growth as evaluated exemplarily with two reference strains (data not shown).

To study growth and biofilm inhibition by the test compounds, samples of precultured pneumococci were diluted in BHI to a McFarland of 0.5 (1.5×10^8 cfu/mL). BHI medium was used for all assays, except for determination of biofilm due to the broad usage of tryptic soy broth (TSB) in pneumococcal biofilm research (Oggioni et al., 2006; Trappetti et al., 2009; Yadav et al., 2012).

Human lung carcinoma cells (A549; Institute of Molecular Virology, University of Münster, Germany) were maintained in Dulbecco's Modified Eagle Medium (Lonza Group Ltd, Basel, Switzerland) supplemented with 10% fetal calve serum (PAA Laboratories GmbH, Cölbe, Germany). Cells were cultivated at 37 °C with 5% CO_2.

Genomic DNA isolation, nanA gene amplification, and sequencing

Genomic DNA of nine *S. pneumoniae* strains was isolated from bacterial cells using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The ~1.5 kb 16S rRNA gene was amplified and sequenced using conventional primers 27F and 1492R devised by Weisburg et al. (1991) to confirm their identity as *S. pneumoniae* (data not shown). NA gene fragments were amplified with PCR Taq Core Kit 10 (MP Biomedicals, Eschwege, Germany). For *nanA* amplification primers were designed based on a pneumococcal *nanA* gene in GenBank (X72967) (Table S1), and for *nanB* and *nanC* we used primers described previously (Burnaugh et al., 2008). Amplification conditions were: 5 min initial denaturation, followed by 38 cycles of 30 s at 96 °C, 30 s at 50–60 °C, and 60 s at 72 °C. Products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by Eurofins Food GmbH (Ebersberg, Germany). The *nanA* of DSM20566 was completely sequenced (GenBank accession number KJ850445).

Preparation of precipitated pneumococcal total proteins for assays

Out of 10 mL of liquid pneumococcal culture in BHI medium, 200 µL were taken to extract the total proteins from cells. Upon addition of 1.8 mL absolute ethanol, the sample was kept overnight at −20 °C. After centrifugation for 10 min, precipitated total protein was washed with ice-cold 70% ethanol and redissolved either in 50 µL PBS buffer for cell-based assay or in 50 µL buffer (32.5 mM MES, 4 mM calcium chloride, pH 6.5) for enzyme-based assays.

Expression and purification of rNanA of S. pneumoniae DSM20566 from E. coli

In order to obtain the *nanA* from *S. pneumoniae* strain DSM20566, primer pair NA_116aaNdeI_fw (5′-TGCACGACATATGGAAAATGTC-3′) and NA_Cter_XhoI_rv (5′-TCAAATCTCGAGAATTCTTCTCT-3′) were applied to amplify this gene. The NdeI/XhoI double-digested PCR product was then ligated to the *E. coli* expression vector pET-28a. The constructed plasmid encodes an N-terminal 6× His-tagged 886aa protein. A 150 mL *E. coli* culture of BL21(DE3)/pTNA20566-116aa was used to synthesize the enzyme. Gene expression was induced by IPTG

at a final concentration of 0.5 mM after a 4 h initial cultivation at 37 °C. The broth was further incubated overnight at 25 °C before harvested by centrifugation. Cell pellet was subjected to B-PER Protein Extraction Reagents (Thermoscientific) for lysis and protein release. N-terminal His-tagged NanA was purified via HisPur Cobalt Spin Column (Thermoscientific) and desalted by Pierce Concentrators PES 30 K MWCO. Protein sample was stored in a 50% glycerol solution at −20 °C.

NA activity and inhibition determination by fluorescence (FL)- and chemiluminescence (CL)-based assays

Pneumococcal NA activity was determined by both FL- and CL-based assays (NA-Fluor™ Influenza Neuraminidase Assay Kit and NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection Kit, respectively, Applied Biosystems) according to the manufacturer's instructions. Some modifications were introduced into the FL-based assay (Okomo-Adhiambo et al., 2010). Briefly, compounds in a ten-fold (FL assay) or half-log (CL assay) serial dilution in the assay buffer (32.5 mM MES, 4 mM calcium chloride, pH 6.5) were mixed (i) with buffer (self-fluorescence control). (ii) a certain dilution of precipitated total protein or (iii) recombinant NanA and incubated at 37 °C for 20 min (Richter et al., 2015). After the addition of 100 μM of MUNANA (2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate) in the FL assay or 5 μL of 500× diluted NA-Star® in the CL assay, the testing plates were incubated for another 2 h and 30 min, respectively, at 37 °C. The reaction was stopped by the addition of 0.1 M glycine, 25% absolute ethanol (pH 10.7) in FL assay. The relative FL of released 4-methylumbelliferone (4-MU) or CL was measured by microplate reader FLUOstar Omega (BMG Labtech GmbH, Ortenberg, Germany). The percentage of self-fluorescence was calculated as described recently (Richter et al., 2015).

At least three individual experiments were performed for the calculation of the IC_{50} values with the JASPR curve fitting software (Okomo-Adhiambo et al., 2010).

Enzyme kinetics

NA enzymatic parameters were evaluated using the FL-based assay as described above. At least three different inhibitor concentrations were applied in combination with four MUNANA substrate concentrations (30, 60, 120, and 240 μM) to determine the K_m value of recombinant NanA of DSM20566 and the K_i value of artocarpin. After 5 min incubation with the enzyme, the reaction was ended by the addition of the stop solution. Relative fluorescence units (RFU) were measured and converted to 4-MU concentration according to the 4-MU standard curve. Velocity (μM/min) of the reaction was represented as the production of 4-MU per minute. The Enzyme Kinetics Module of SigmaPlot 12.0 (Systat Software, San Jose, CA) was applied to fit the data to the Michaelis–Menten equation using nonlinear regression and determine the enzymatic activity (V_{max}) and the inhibition constant (K_i) of NAI.

Analysis of NA activity and its inhibition in lectin-based hemagglutination (HA) assay

The HA assay (Nakano et al., 2006; Pereira, 1983) was adapted to evaluate the pneumococcal NA activity as well as its inhibition by NAI (Richter et al., 2015).

To evaluate NA activity of pneumococci, 5 μL of a 25% human erythrocyte suspension (in PBS buffer) was subjected to 100 μL of serial diluted (dilution factor 2) precipitated pneumococcal total protein in flat-bottom 96-well polystyrene microplates and incubated at 37 °C in 5% CO_2 for 4 h. Afterwards, 20 μL of this protein-erythrocytes suspension were added to equal volume of *Arachis hypogaea* (Sigma-Aldrich GmbH) lectin (50 μg/mL) in V-shaped 96-well polystyrene microplates and incubated at 4 °C overnight. Thereafter, the lowest concentration of precipitated pneumococcal total protein causing hemagglutination was visually determined (mean value of three independent assays) and used as readout for NA activity.

To analyze NA inhibition by test compounds, 50 μL of the determined bacterial protein dilution was added to 50 μL of serial compound dilutions (dilution factor 2) or PBS (NA activity control) in a flat-bottom 96-well polystyrene microplate. After incubation at 37 °C and 5% CO_2 for 20 min, 5 μL of 25% erythrocytes solution was supplemented to the protein-compound suspensions, followed by carefully homogenization and incubation under the same condition for 4 h. Then NA activity was determined as described above via hemagglutination. Negative controls included mere erythrocytes in PBS, a mixture of erythrocytes and bacteria proteins, and a mixture of erythrocytes and lectin. In addition, a cocktail of erythrocytes, lectin, and compounds without bacterial protein (one well per compound concentration) was included to confirm that hemagglutination was not caused by the interaction between compounds and erythrocytes. The compound concentration that prevented hemagglutination was defined as minimal NA inhibitory concentration. Each compound was tested at least three times.

Erythrocyte hemolysis assay

A standard hemolysis assay was performed with human erythrocytes (Yu et al., 2011) with some modifications. Briefly, 10 μL of 25% diluted erythrocytes in PBS were incubated with 200 μL of serial diluted artocarpin (diluted in PBS) for 3 h at 37 °C. Final concentrations of artocarpin used were 100 μM, 31.6 μM, 10 μM, 3.2 μM, 1 μM, and 0.3 μM. After that, the mixture was gently shaken and centrifuged at 1500 × *g* for 10 min. 100 μL of supernatant from each sample was transferred to a flat-bottom 96-well plate. The absorbance value of hemoglobin at 577 nm was measured with the reference wavelength at 655 nm. Erythrocytes diluted in PBS or in aqua bidest were used as negative and positive control, respectively. The percent of hemolysis was calculated as: hemolysis (%) = [(sample absorbance − negative control)/(positive control − negative control)] × 100%.

Determination of MIC_{90} and MBC

For broth microdilution assay, pneumococci of McFarland 0.5 (1.5×10^8 cfu/mL) were 50×diluted in BHI, as well as test compound in a two-fold serial dilution in BHI. 100 μL of diluted substance was mixed with 50 μL bacteria in a 96-well V-shape plate in two repetitions and incubated overnight at 37 °C with 5% CO_2. Wells without compound were used as positive growth control and wells without bacteria as negative control for medium sterility. The maximal concentrations of compounds in test were 200 μM for oseltamivir and DANA, and 50 μM for artocarpin, katsumadain A, and rifampicin. The growth of bacteria was monitored by measuring optical density at 620 nm (OD_{620}) at 0 h and 18 h. Before calculating the minimal inhibitory concentration (MIC_{90}), the OD_{620}-0 h was subtracted from OD_{620}-18 h. The mean value of positive growth control was set as 100%. MIC_{90} was defined as the drug concentration that reduced 90% turbidity of the untreated bacterial suspension.

Bactericidal effect of the compounds was investigated using the overnight grown MIC plates. 100 μL of suspension from each well was subcultured on blood agar plates without test agent at 37 °C with 5% CO_2. The minimal bactericidal concentration (MBC) was defined as the lowest concentration that reduced the viability of the initial bacterial inoculum by ≥99.9%. Antibacterial agents are

usually regarded as bactericidal if the MBC is not more than four times the MIC (French, 2006).

Biofilm inhibition assay

Broth was diluted as described for broth microdilution assay in TSB and incubated for 2 h at 37 °C with 5% CO_2 in 96-well F-bottom plates. Then, the supernatant was replaced by 200 μL of fresh medium without compounds as positive control, or by 150 μL of fresh medium plus 50 μL of two-fold serial dilution of compound (maximal 50 μM; each concentration in duplicate). Six wells without bacteria were used as negative controls. After 24 h incubation, plates were washed with water and stained with crystal violet overnight. After rinsing the plates with water, the crystal violet was eluted with 150 μL of lysis buffer (0.898 g of sodium citrate and 1.25 mL of 1 M HCl in 98.05 mL 47.5% ethanol) (Schmidtke et al., 2001), and the optical density of the elution was measured at 550 nm (OD_{550}). Minimal biofilm inhibitory concentration ($MBIC_{90}$) was defined as the drug concentration reducing the OD_{550} of untreated controls by 90%.

Adherence inhibition assay

Bacterial adherence was quantified based on Parker's method (Hsiao et al., 2009). Pulmonary epithelial cell A549 monolayer were grown for 3 days on 24-well tissue culture plates or 8-well plastic slides (Thermoscientific) until confluence (Singer et al., 2010). Suspensions of the strain DSM20566 and PN8828 grown in BHI broth to mid-exponential phase were added (multiplicity of infection (MOI) of 10) to A549 monolayer for 1.5 h at 37 °C and 5% CO_2 in the absence (control) or presence of the compounds. After vigorous washing for three times with PBS, the cells in the 24-well plate were lysed with 0.5% saponin (Sigma-Aldrich GmbH) for 5 min. The cells on the plastic slide were fixed with methanol for 10 min. Aliquots of saponin-detached cells were serially diluted and quantitatively plated on Columbia blood agar plates. The numbers of adherent bacteria on the epithelial cells were determined as colony forming units (CFU) from dilution counts. The mean value of CFU from control wells was set as 100%. Adherence inhibitory concentration (AIC_{50}) was defined as the drug concentration reducing the adherence by 50%. In parallel, compound-treated pneumococci were subcultured on blood agar plates to make sure that the reduced adherence was not due to the bactericidal effect of the compounds. The slide was gram-stained according to a standard protocol in the University Hospital Jena and examined with a Zeiss Axio Imager A1 microscope at 100-magnification.

Determination of cytotoxicity of the compounds

The cytotoxicity of compounds was determined with three-day-old confluent A549 cell monolayers grown in 96-well F-bottom plates as described previously (Schmidtke et al., 2001). Briefly, cells were incubated with serial two-fold dilutions of compound at 37 °C in 5% CO_2 atmosphere for 72 h (each concentration in duplicate); cells without compound treatment served as basic controls. The Dynex Immuno Assay System (DIAS, Guernsey, Great Britain) was afterwards applied to gently wash, fix and stain the cells with crystal violet dye solution in methanol, formalin and water. Subsequently, the crystal violet was eluted by 100 μL of lysis buffer (see above) for the measurement of OD_{550}. Cell viability was evaluated as the percentage of the mean value of optical density resulting from the six cell controls, which was set 100%. The 50% cytotoxic concentrations (CC_{50}) were calculated from the mean dose–response curves of at least three assays each with two parallels.

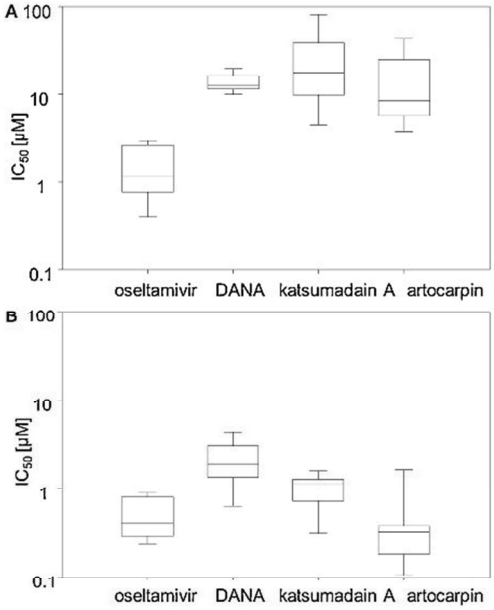

Fig. 1. Box and whisker plots showing distribution of mean 50% inhibitory concentration (IC_{50}) values of NAIs oseltamivir, DANA, katsumadain A, and artocarpin among the nine studied pneumococci strains. After confirming activity of precipitated bacterial proteins, they were used in the (A) FL and (B) CL enzyme inhibitory assay. Mean IC_{50} values are calculated based on a minimum of three individually performed experiments.

Results

All S. pneumoniae strains tested encoded NA genes and exhibited NA activity

As shown in Table 1, we successfully amplified and sequenced *nanA* fragments from the genomic DNAs of the three pneumococcal reference strains and the six clinical isolates tested in this study. All reference strains and five of the six clinical isolates also encoded *nanB* (with the exception of strain CF6937). In only two of the studied clinical isolates *nanC* was detected but in none of the reference strains.

In all tested strains, significant NA activity was detected by the CL, FL and HA assays for ethanol extracts of total bacterial protein (Table 1). In order to confirm these results and prepare for subsequent studies on enzyme inhibition, we exemplarily expressed NanA of strain DSM20566 in *E. coli*, for which we were able to detect activity in all three assays.

Inhibitory effect of investigational NAIs on pneumococcal NA

When assessing the inhibition of NA activity of the precipitated total proteins of all strains, we found that oseltamivir and DANA, as well as the two natural compounds artocarpin and katsumadain A, exhibited their inhibitory potential in a dose-dependent manner (Fig. 1A, Table 2). In contrast, zanamivir did not show an effect. The mean IC_{50} value determined by the FL-based assay for oseltamivir was 1.40 μM, whereas ~10-fold higher concentrations of DANA and artocarpin were required to produce a similar effect (Fig. 1A, Table S2). The IC_{50} values of katsumadain A were in a broad range between 4.50 and 81.03 μM, most probably caused by the strong self-fluorescence determined for this compound (Richter

Table 2
Inhibition of pneumococcal NA activity (in lectin-based HA assay).

Compounds	Inhibitory concentration (μM)[a]		
	DSM20566	D39	CJ9400
Oseltamivir	2.1 ± 1.3	10.0 ± 0.0	0.3 ± 0.0
Zanamivir	>100	>100	>100
DANA	45.3 ± 30.6	>100	15.4 ± 10.8
Katsumadain A	3.2 ± 0.0	1.0 ± 0.0	0.7 ± 0.4
Artocarpin	7.7 ± 4.0	–[b]	2.6 ± 1.1

[a] Mean inhibitory concentration values were calculated based on three individually performed assays.
[b] The erythrocytes were lysed in the presence of artocarpin at concentrations higher than 10 μM.

et al., 2015). All other studied compounds did not exhibit self-fluorescence effects (data not shown).

The CL-based assay was used to confirm the results from FL assays. Our results underline susceptibility of NanAs of all nine strains for oseltamivir, DANA, katsumadain A, and artocarpin (Fig. 1B, Table S3), as well as inefficacy of zanamivir (data not shown). Generally, IC_{50} values determined by CL-based assays are lower than those obtained from FL-based assays. Artocarpin and oseltamivir exhibited mean IC_{50} values of 0.49 μM and 0.51 μM, respectively. The broad range of inhibitory effects of katsumadain A measured in FL-based assays towards diverse test strains was reduced to 0.31–1.61 μM in the CL-based assays (Fig. 1B).

To validate bacterial enzyme inhibition by the test compounds under more physiological conditions, the cell-based HA assay was performed with three selected pneumococcal strains, DSM20566, D39 as reference strains and one clinical isolate, CJ9400. As shown in Table 2 the total protein extracted from the DSM20566 and CJ9400 strains was susceptible to all interrogated NAIs in this cell-based assay.

With the exception of katsumadain A, the tested NAI showed lower activity against the reference strain D39, or even no activity in the case of DANA and artocarpin. In general, the activities of the cell-based HA assay clearly confirmed the results obtained from the enzyme-based assays (Tables 2 and 3). A 20% hemolysis was detected when 31.60 μM of artocarpin was applied to erythrocytes (Fig. S2).

NAI susceptibility, kinetic properties, and the mechanism of inhibition of the pneumococcal rNanA

To confirm that the IC_{50} values determined by using precipitated total pneumococci proteins correspond with inhibition of the ubiquitously expressed pneumococcal NanA, *E. coli*-derived rNanA of reference strain DSM20566 was tested in the assays described above. As shown in Tables S2 and S3 the susceptibilities of the precipitated total protein and the recombinant NanA towards four test agents were in a comparable range.

Enzyme kinetics revealed a substrate affinity (K_m) of rNanA for 4-MUNANA of 23.60 μM. The inhibition constant of oseltamivir and artocarpin was determined and the mechanism of inhibition modeled. As expected, influenza virus specific NAI oseltamivir competitively inhibited the activity of rNanA with a K_i value of 0.23 μM (Fig. 2A). According to the SigmaPlot analysis, artocarpin inhibition is of a mixed type with an inhibition constant K_i of 9.70 μM (Fig. 2B). With increasing concentration of artocarpin, the K_m of rNanA increases and V_{max} drops, indicating that artocarpin binds to the free rNanA and the rNanA–MUNANA complex with different affinity. We were unable to determine the mechanism of inhibition for katsumadain A due to its strong self-fluorescence under the FL-assay conditions (Richter et al., 2015).

Antipneumococcal effects of artocarpin

Since NanA was described to be involved in biofilm formation (Brittan et al., 2012; Parker et al., 2009), we investigated the

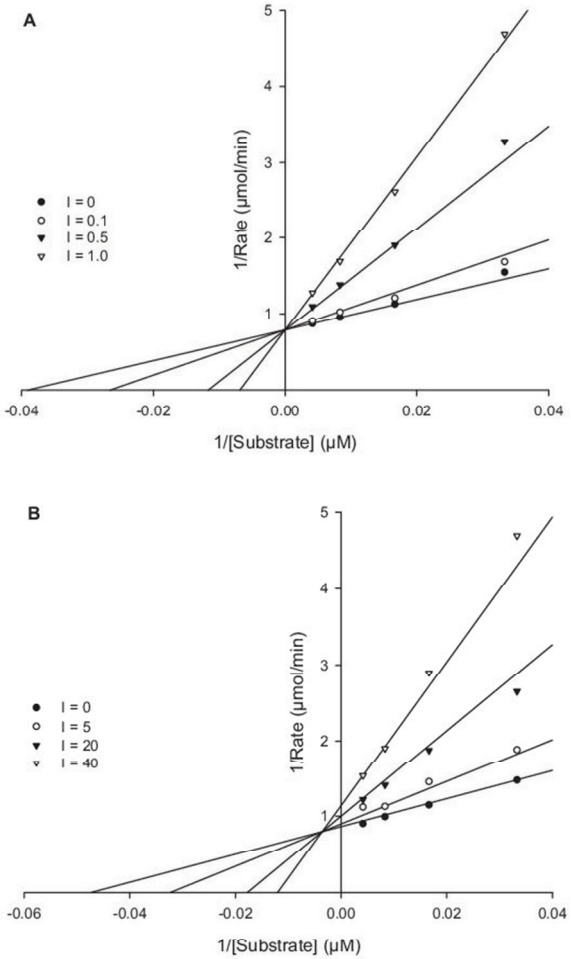

Fig. 2. Lineweaver–Burk plot analysis of the inhibition mechanism of NAI. (A) Competitive inhibition of oseltamivir at concentrations of 0.0, 0.1, 0.5 and 1 μM. Regardless of the presence of oseltamivir, V_{max} is invariant, however, the increase in concentration of oseltamivir led to the raise of K_m. (B) Mixed inhibition of artocarpin at concentrations of 0, 5, 20 and 40 μM. The presence of artocarpin resulted in a decreased V_{max} and increased K_m. The K_i values of oseltamivir and artocarpin are 0.23 ± 0.04 μM and 9.68 ± 1.49 μM, respectively.

Table 3
Mean 90% inhibitory concentration and standard deviation of oseltamivir, DANA, artocarpin, and katsumadain A against three reference strains and six clinical isolates in broth microdilution and biofilm assay as well as bactericidal concentrations.

90% Minimal inhibitory concentration (μM)[a]										
Compound	DSM20566	D39	DSM14378	CJ9400	CF6937	BC7326	CF8919	PN8828	BC57
Rifampicin	0.041 ± 0.011	0.021 ± 0.002	0.031 ± 0.023	0.032 ± 0.030	0.034 ± 0.028	0.016 ± 0.005	0.009 ± 0.001	0.005 ± 0.001	0.012 ± 0.002
Oseltamivir, zanamivir, DANA, katsumadain A	>50	>50	>50	>50	>50	>50	>50	>50	>50
Artocarpin	5.58 ± 0.97	1.29 ± 0.31	5.75 ± 0.13	3.45 ± 2.05	3.39 ± 2.10	1.12 ± 0.37	1.45 ± 0.01	0.99 ± 0.48	1.45 ± 0.04
90% Minimal biofilm inhibitory concentration (μM)[a]									
Rifampicin	0.049 ± 0.030	0.013 ± 0.002	0.014 ± 0.008	0.003 ± 0.001	0.020 ± 0.002	0.016 ± 0.010	0.006 ± 0.004	0.011 ± 0.007	0.012 ± 0.001
oseltamivir, zanamivir, DANA, katsumadain A	>50	>50	>50	>50	>50	>50	>50	>50	>50
Artocarpin	2.88 ± 0.07	2.22 ± 0.76	2.37 ± 0.67	2.97 ± 0.13	2.73 ± 0.27	2.02 ± 0.62	1.15 ± 0.34	1.40 ± 0.07	2.35 ± 0.68
Bactericidal concentration (μM)[b]									
Rifampicin	1.97 ± 1.23	0.22 ± 0.09	0.14 ± 0.06	0.23 ± 0.16	0.05 ± 0.03	0.08 ± 0.03	not studied	not studied	not studied
Oseltamivir, zanamivir, DANA, katsumadain A	>50	>50	>50	>50	>50	>50	not studied	not studied	not studied
Artocarpin	3.13 ± 0.00	2.50 ± 0.77	4.69 ± 1.98	2.74 ± 0.68	3.44 ± 1.53	3.32 ± 1.94	not studied	not studied	not studied

[a] The IC_{50} values and standard deviations were calculated of at least three independent tests with each concentration tested in duplicate.
[b] The bactericidal concentrations were calculated of at least two independent tests.

capacity of NAIs to reduce the biofilm production of *S. pneumoniae*. No significant inhibition on biofilm formation was observed upon addition of oseltamivir, zanamivir, DANA, or katsumadain A up to 50 μM. However, artocarpin exhibited a strong dose-dependent effect, with mean $MBIC_{90}$ values in the range of 1.15 to 2.97 μM (Table 3). As an example, the dose–response curve of reference strain DSM20566 is reported in Fig. 3A.

In contrast to all other tested NAIs, artocarpin also inhibited planktonic growth of all tested pneumococcal strains (Fig. 3B and Table 3). Its MIC_{90} values, determined by the broth microdilution assay, ranged from 0.99 to 5.75 μM. The MIC_{90} values of the antibiotic rifampicin (used as positive control) were in a range from 0.01 to 0.04 μM.

We also detected significant bactericidal activity for artocarpin and the control antibiotic rifampicin. Both compounds reduced the viability of studied pneumococci by a factor of >1000 within 18 h. Neither oseltamivir nor DANA or katsumadain A exhibited inhibitory or bactericidal activity at concentrations up to 50 μM (the maximum tested concentration; Table 3).

Artocarpin prevents pneumococcal adhesion to pulmonary epithelial cells without cytotoxic effects

One of the main functions of pneumococcal NA is desialylation of sugar moieties on the cell surface, serving as receptors for bacterial attachment to airway epithelial cells (Brittan et al., 2012). After confirming the inhibitory capacity of viral NAIs for pneumococcal NA, we explored their effect on pneumococcal adherence to pulmonary epithelial A549 cells. This was studied using an adherence assay with DSM20566 and PN8828 (MOI of 10). Artocarpin led to a 50% reduction in adherence levels of the reference strain and clinical isolate to the cell culture at 7.50 and 4.10 μM, respectively (Fig. 4A, Table S4). Similar effects of the reduced bacterial adherence on lung cells with artocarpin were also observed via Gram staining (Fig. 4B). None of the other tested NAIs showed any effect on the adhesion of pneumococci (Table S4).

To confirm that the adherence inhibitory effect was not a result of the bactericidal potential of the test compounds, DSM20566 was incubated in the absence or presence of artocarpin within 1.5 h and then cultivated on blood agar. No bactericidal effect was observed (results not shown). Furthermore, artocarpin did not affect cell viability.

To further analyze the compatibility of artocarpin with A549 cells, we monitored the viability of confluent cell monolayers for 72 h of coincubation with the compound. As reported in Fig. 5A and B, artocarpin exhibited a low level of cytotoxicity, with a CC_{50} of 38.00 μM. At a concentration of 12.50 μM, the effective concentration for adherence inhibition, no cytotoxic effect was observed. This was further confirmed by microscopic analysis (Fig. 5B).

Discussion and conclusion

Bacterial NAs have been implicated in the pathogenesis of *S. pneumoniae* and lethal synergism during influenza infections, and were suggested as promising drug targets (Taylor, 1996). Our current work demonstrated katsumadain A and artocarpin as inhibitors of pneumococcal NA. In addition, artocarpin exhibited antipneumococcal activity.

As one of the most conserved gene loci of *S. pneumoniae*, *nanA* was detected in all strains described previously (Kelly et al., 1967; Pettigrew et al., 2006) and in all nine strains investigated in the present study. In addition, *nanB* was detected for eight, and *nanC* for two strains. In agreement with NA gene detection, pronounced NA activities were observed from the precipitated total protein of all strains and in the *E. coli*-synthesized recombinant

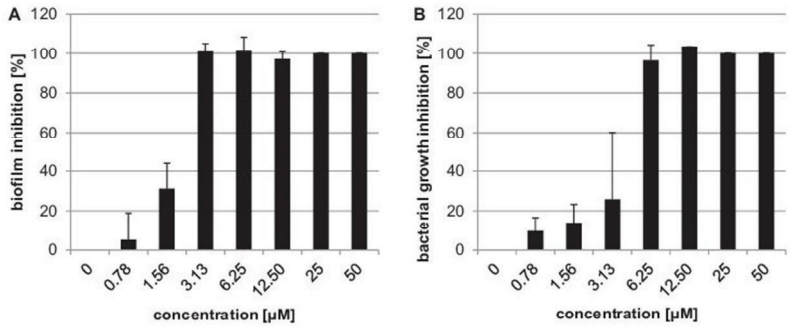

Fig. 3. Susceptibility of pneumococcal reference strain DSM20566 to artocarpin. (A) The inhibition of biofilm formation, and (B) of bacterial growth by artocarpin is shown. Bars represent the mean and standard deviation of at least three assays each with two parallels per concentration.

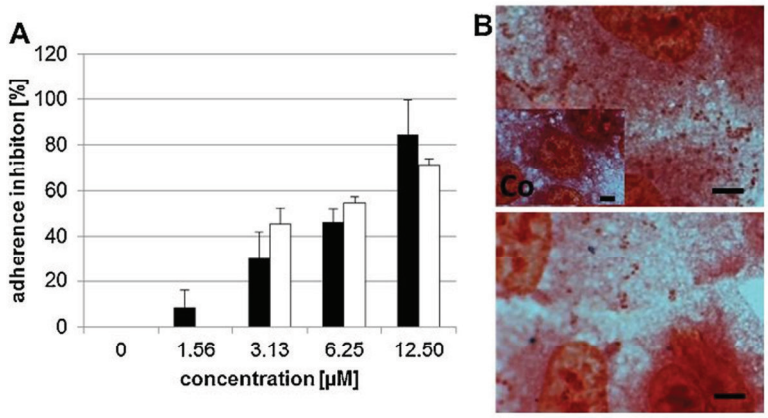

Fig. 4. Artocarpin inhibits the adherence of pneumococci to A549 cells. (A) Percentage of inhibition of adherence of pneumococcal reference strain DSM20566 (black) and clinical isolate PN8828 (white) by artocarpin in comparison to untreated bacteria. Mean and standard deviation of at least three assays each with two parallels per concentration are shown. (B) Gram staining was used to confirm adhesion of strain DSM20566 in the absence (upper image) and presence (down image) of 12.5 µM artocarpin. The untreated cell control (Co) is shown in the left lower corner of the upper image.

NanA of strain DSM20566. The catalytic activity of all these strains was effectively blocked by oseltamivir and DANA, as well as the influenza A virus NAIs katsumadain A and artocarpin (Grienke et al., 2010; Grienke et al., 2012) at low micromolar concentrations in three different types of NA inhibition assays. However, the inhibitory activity of oseltamivir on bacterial NAs was found to be lower by a factor of 1000 when compared to viral NA (K_i of 0.32 nM) (Collins et al., 2008). The inhibitory effect of NAI was corroborated using purified recombinant rNanA as target. The IC_{50} values obtained for the competitive, virus-specific NAIs DANA and

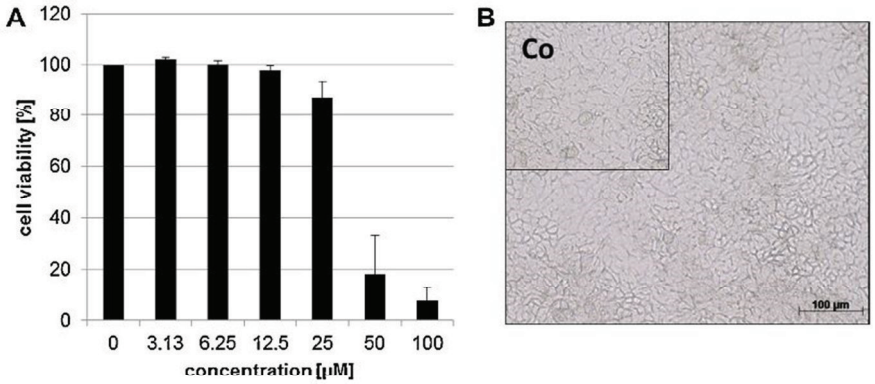

Fig. 5. The influence of artocarpin on the viability of A549 monolayer's during 72 h of incubation. (A) Mean percentage of cell viablilty (OD of untreated cell control was set 100%) and standard deviation of at least three cytotoxicity assays each with two parallels per concentration are shown.) (B) Photography of phase-contrast microscopy of cells incubated for 72 h with 12.5 µM artocarpin. The untreated cell control (Co) is shown in the left upper corner. Bar represents 5 µm.

oseltamivir are in good agreement with published data (Gut et al., 2011).

The enzyme kinetics study with recombinant NanA of strain DSM20566 revealed a competitive mechanism of inhibition for oseltamivir, and a mixed inhibition for artocarpin. Oseltamivir is known to bind the substrate binding site of NanA (Gut et al., 2011), and this is confirmed by the enzyme kinetics measured in this work. The inhibition mechanism of artocarpin as suggested by nonlinear regression (SigmaPlot) is a mixed type, meaning that artocarpin binds, with different affinity, to both free NanA and NanA–sialic acid complexes. This suggests the presence of an alternative binding site for artocarpin, different from the natural substrate recognition site.

Previous reports on the key role of NanA in the bacterial adhesion to host cells, biofilm formation and disease (Nobbs et al., 2009), and the herein reported inhibition of pneumococcal NA by oseltamivir, DANA, katsumadain A and artocarpin prompted us to study their potential to reduce the adhesion of *S. pneumoniae* to host cells or tissues, and to block the formation of the microbial structured community. Intriguingly, oseltamivir, DANA and katsumadain A did not reduce pneumococcal adherence to A549 cells, and did not inhibit bacterial growth or biofilm formation, despite their significant inhibitory activity on NAs. To our knowledge, this is the first report on antipneumococcal activity of NAIs, although it has previously been demonstrated that *N*-acetyl neuraminic acid inhibits biofilm formation (Parker et al., 2009). It remains unclear why oseltamivir, DANA, or katsumadain A does not affect pneumococcal adhesion, growth, and/or biofilm formation, despite inhibiting NA activity at low micromolar concentrations. It could be that under physiological conditions compound binding is not strong enough to compete with natural substrates. Another possibility is that blockage of NA activity by binding of the compound to the cleavage site does not result in a measurable inhibitory effect. This would contradict with some previous studies (Brittan et al., 2012; Parker et al., 2009), but be in agreement with the results of King and colleagues (King et al., 2004, 2006) obtained for NA deletion mutants of pneumococci. King and colleagues did not detect a reproducible difference between the percentage of adherent wild-type and nanA knockout D39 pneumococci, and for the level of persistence of murine colonization.

Artocarpin significantly reduced the adherence to epithelial cells A549 and exhibited antipneumococcal activity on planktonic bacteria as well as biofilm formation at low micromolar concentrations. Unlike the other NAIs tested in this study, but similar to the control antibiotic rifampicin, artocarpin showed bactericidal activity. Thus, in contrast to all other studied NAIs, artocarpin exerted antipneumococcal activity. This difference might be based on the mixed binding mode of artocarpin to pneumococcal NA, or activity on (a) further pneumococcal target(s).

There is not enough evidence to conclude whether or not the inhibition of pneumococcal growth, adherence and biofilm formation by artocarpin is to be entirely ascribed to the inactivation of NanA or a result of additional interaction between artocarpin and one or more additional pneumococcal targets.

To the best of our knowledge, this is the first report of a compound with strong inhibitory activity on pneumococcal NAs that in addition is effective in reducing bacterial adherence to pulmonary epithelial cells, biofilm formation, pneumococcal growth, and viability. Strong antipneumococcal and NA inhibitory activity render artocarpin a potentially interesting lead in the search for dual acting antipneumococcal drugs.

Financial disclosure

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2014.12.004.

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Supplementary

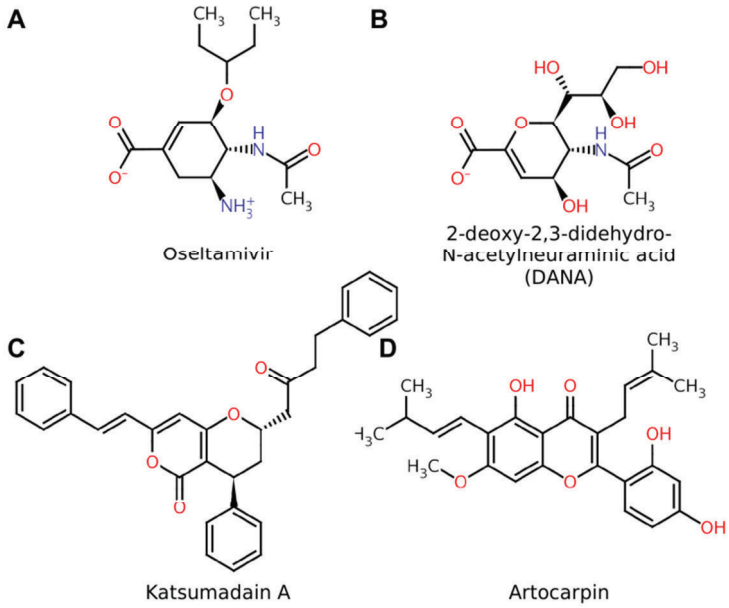

Figure S1. Structure of the NAIs. Names and abbreviations as used in this study. Protonation state of the molecules corresponds to the major species at pH 7.

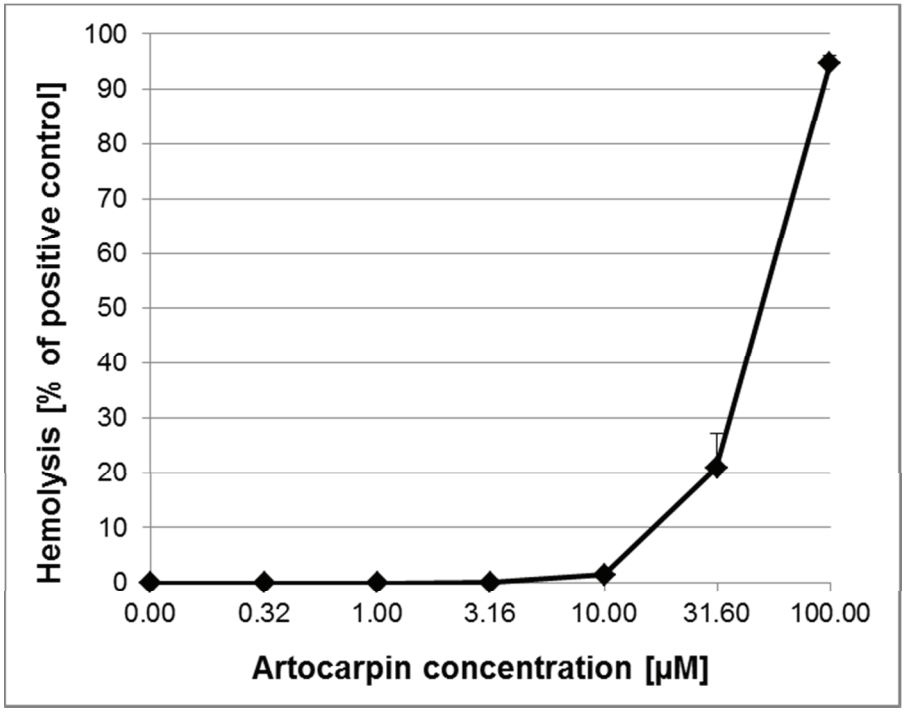

Figure S2. Relative rate of hemolysis in human erythrocytes is shown upon incubation with different concentrations of artocarpin. The presence of hemoglobin in the supernatant (red) was measured at 577 nm with the reference wavelength at 655 nm. Erythrocytes diluted in PBS or in aqua bidest were used as negative or positive control, respectively. Data were mean ± SD (n = 3).

Table S1. PCR primers used in this study.

Primer	Sequence (5' to 3')	Binding site (bp) [a]
F1f	GTCATTTAAATGAACTGTAGTAAAAG	-67-(-41)
F1r	CCAGATTTCAGACTTGTTCGAG	743-764
F2f	GCACCCTTAAAAGTTAAACCAGGTC	631-655
F2r	GGTTTAACAGGATCTACAACAACG	1476-1499
F3f	GCTTATACCATTCGAGAAAATGG	1414-1436
F3r	CTCACCATTTTCTTCGACACGTG	2186-2208
F4f	CACAGGTTAAAGATGTCTATGTTC	2066-2089
F4r	AGACCTCTTATGATTTCGTATCAGC	87-111
F1iF	CCAAGAGATTACTATGCACGAGATTTG	319-345
F2iF	GATTCGGAATCTCACTGTGTATAATC	855-880
F3iR	CGAAGTACAATTCCTGTTCCAGGAC	1706-1730
F4iR	CGATTTCATGAACAGCTGGCTCTG	2900-2923

[a] Position within R36A (ancestral strain D39) *nanA* sequence (accession number X72967).

Table S2. Mean 50% inhibitory concentration and standard deviation of oseltamivir, DANA, artocarpin, and katsumadain A, measured against the precipitated pneumococcal protein in the FL-based assay.

Strains	FL inhibition assay IC_{50} (µM) [a]			
	oseltamivir	DANA	katsumadain A	artocarpin
DSM20566	0.91 ± 0.14	10.62 ± 0.85	15.24 ± 2.99	8.43 ± 4.08
recombinant NanA	2.85 ± 0.89	18.03 ± 1.87	36.79 ± 24.16	9.96 ± 5.79
D39	2.89 ± 0.09	13.79 ± 1.82	17.28 ± 3.57	6.06 ± 0.77
DSM14378	0.98 ± 0.22	12.17 ± 2.65	45.57 ± 7.14	9.74 ± 2.58
CJ9400	0.40 ± 0.09	19.23 ± 4.01	81.03 ± 14.69	44.83 ± 12.34
CF6937	1.43 ± 0.26	16.42 ± 2.39	13.93 ± 0.93	3.83 ± 2.13
BC7326	1.14 ± 0.54	9.92 ± 1.37	32.57 ± 17.08	11.71 ± 5.66
CF8919	2.33 ± 1.38	12.38 ± 2.96	5.79 ± 0.70	7.52 ± 3.11
PN8828	0.62 ± 0.06	15.44 ± 9.06	33.97 ± 6.60	36.08 ± 21.11
BC57	2.85 ± 1.16	12.41 ± 0.52	4.50 ± 1.58	5.48 ± 1.71

[a] IC_{50} values and their standard deviations were calculated for a minimum of three independent tests.

Table S3. Mean 50% inhibitory concentration and standard deviation of oseltamivir, DANA, artocarpin, and katsumadain A, measured against the precipitated protein in the CL-based assay.

strains	CL inhibition assay IC_{50} (µM) [a]			
	oseltamivir	**DANA**	**katsumadain A**	**artocarpin**
DSM20566	0.73 ± 0.15	2.43 ± 0.76	1.01 ± 0.37	0.33 ± 0.14
recombinant NanA	0.93 ± 0.42	3.16 ± 0.87	0.52 ± 0.12	0.30 ± 0.09
D39	0.85 ± 0.15	3.61 ± 1.85	1.25 ± 0.44	0.32 ± 0.02
DSM14378	0.35 ± 0.05	1.93 ± 0.83	1.61 ± 1.06	0.43 ± 0.18
CJ9400	0.24 ± 0.08	4.27 ± 2.83	1.31 ± 0.26	0.33 ± 0.10
CF6937	0.60 ± 0.13	2.05 ± 0.18	1.18 ± 0.16	1.65 ± 0.11
BC7326	0.33 ± 0.16	1.03 ± 0.29	0.31 ± 0.06	0.11 ± 0.05
CF8919	0.40 ± 0.10	0.62 ± 0.14	0.65 ± 0.12	0.16 ± 0.03
PN8828	0.25 ± 0.12	1.68 ± 0.52	0.31 ± 0.06	0.77 ± 0.14
BC57	0.88 ± 0.45	1.88 ± 0.76	1.14 ± 0.19	0.33 ± 0.12

[a] The IC_{50} values and standard deviations were calculated of at least three independent tests.

Table S4. Absolute numbers of adherent bacteria (CFU/well) in the adherence inhibition assay.

	Adherence inhibition assay CFU/well × 10^6		
compound	**conc. in µM**	**DSM20566**	**PN8828**
no compound		20.13 ± 13.51	0.07 ± 0.04
oseltamivir	50.00	16.04 ± 14.33	not tested
	25.00	17.71 ± 15.07	not tested
DANA	50.00	12.96 ± 9.44	not tested
	25.00	16.58 ± 12.72	not tested
katsumadain A	50.00	18.63 ± 17.58	not tested
	25.00	16.88 ± 13.71	not tested
artocarpin	12.50	4.83 ± 2.72	0.02 ± 0.01
	6.25	8.43 ± 9.23	0.03 ± 0.02
	3.13	13.50 ± 9.01	0.04 ± 0.03

2.3 Publikation 3

Titel: Pneumococcal neuraminidases as key players in the lethal synergism with influenza viruses

Zeitschrift: Cell Host and Microbe

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Zusammenfassung:
Durch die strukturelle Ähnlichkeit viraler und bakterieller NA besitzen Pneumokokken-NA (NanA, NanB) das Potenzial, die Freisetzung von Influenzaviren zu unterstützen. Im *In-vitro*-Modell mit einem Influenzavirus und rekombinanter NanA (rNanA) oder rekombinanter NanB (rNanB) in Lungenepithelzellen wurde der Effekt der NAI auf die Virusausbreitung und den Virusertrag in Ab- und Anwesenheit der bakteriellen NA verglichen. Es zeigte sich, dass nur NanA, und nicht NanB, die Funktion der viralen NA übernehmen kann und so auch nur die NanA eine Rolle im letalen Synergismus von Influenzaviren und Pneumokokken einnimmt.

Pneumococcal neuraminidases as key players in the lethal synergism with influenza viruses

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SUMMARY

Co-infections with *Streptococcus pneumoniae* exacerbate disease and mortality during influenza epidemics and pandemics. In contrast to viral factors, the effect of bacteria to this lethal synergism is less studied. Our results confirm that pneumococcal neuraminidases cleave cellular α2.3-linked (NanA and NanB) and/or α2.6-linked (NanA) sialic acids. High amounts of both neuraminidases hinder replication of the A(H1N1)pdm09 isolate A/Jena/8178/09 by removing its receptors. However, low amounts of pneumococcal neuraminidases significantly increase virus spread and yield in A549 cells and have also the potential to rescue viral replication from inhibition by neuraminidase inhibitors such as zanamivir. These results explain why synergism only occurs when the viral infection precedes bacterial exposure and why virus titers raise after co-infection. We demonstrate that dual neuraminidase inhibitors, targeting viral and pneumococcal neuraminidases, might overcome this fatal synergistic function of pneumococcal neuraminidases.

Running title: Pneumococcal neuraminidases and influenza

Key words: pneumonia, co-pathogenesis, antibacterial, antiviral, microbial communication neuraminidase inhibitors

INTRODUCTION

Streptococcus pneumoniae (*S. pneumoniae*) colonizes the nasopharynx of 70% of children younger than 10 years and of ~8% of the older people (Bogaert et al., 2004). From the 94 serotypes known (Song et al., 2013), serotypes 1, 5, 6A, 6B, 14, 19F and 23F account for the majority of invasive *S. pneumoniae* diseases such as septicemia, meningitis, and pneumonia (Mehr et al., 2012). Moreover, secondary *S. pneumoniae* infections exacerbate pneumonia in influenza patients leading to high mortality in influenza epidemics and pandemics based on co-pathogenesis of both pathogens, also called lethal synergism, reviewed in (McCullers, 2014). In 1918, the influenza pandemic killed an estimated 40 to 50 million people, with bacterial pathogens (mostly *S. pneumoniae*) implicated in more than 95% of all severe cases of influenza and deaths. Co-infection with *S. pneumoniae* was also correlated with the severity of the H1N1 pandemic influenza in 2009 (Louie et al., 2009; Palacios et al., 2009). Several viral factors, in particular the viral neuraminidase (NA) contributing to the lethal synergism by providing attachment receptors and nutrients to the bacteria, were thoroughly investigated (McCullers, 2004; Peltola et al., 2005; Siegel et al., 2014). In contrast, the role of *S. pneumoniae*-specific virulence factors was less studied until now. Rebound virus titers after co-infection with *S. pneumoniae in vivo* (Chockalingam et al., 2012; McCullers et al., 2002; Smith et al., 2013) implicate a role of the bacteria for virus release. The mechanism is unclear, reviewed in (McCullers, 2014) but rescue of influenza virus replication *in vitro* from inhibition by the NAI zanamivir in presence of *S. pneumoniae* culture supernatant (Nishikawa et al., 2012) suggests a role of bacterial NAs in the *in vivo* observed rebound virus titers.

S. pneumoniae express three distinct NAs: NanA, NanB, NanC (Pettigrew et al., 2006; Xu et al., 2011). NanA, the most active and highly expressed one (Berry et al., 1996; Manco et

al., 2006), was detected in all *S. pneumoniae* strains and has a conserved catalytic site (King et al., 2005; Pettigrew et al., 2006; Walther et al., 2015). NanA acts as a sialidase, hydrolyzing α2-3-, α2-6- and α2-8-sialyllactose to release N-acetyl-neuraminic acid (Neu5Ac) (Xu et al., 2011). The enzyme is required for colonization and sepsis *in vivo* (Manco et al., 2006; Orihuela et al., 2004; Song et al., 2008). Its impact on biofilm formation is in discussion (King et al., 2004; Oggioni et al., 2006; Parker et al., 2009; Trappetti et al., 2009; Walther et al., 2015). NanB, detected in most but not all *S. pneumoniae* strains (Pettigrew et al., 2006; Walther et al., 2015), represents a trans-sialidase transferring 2,7-anhydro-Neu5Ac from preferentially α2-3-sialosides to other glycoconjugates (Gut et al., 2008) and contributing to deglycosylation of sialic acids *in vitro* (Burnaugh et al., 2008) and *in vivo* (Siegel et al., 2014; Tong et al., 2001). NanB deficient strains are unable to colonize the nasopharynx or to cause sepsis (Manco et al., 2006). Furthermore, NanB is highly expressed in biofilm *in vitro* and during pneumonia in a mouse model (Manco et al., 2006; Oggioni et al., 2006). Different pH optima (pH 6.5 for NanA, pH 5.0 for NanB) (Berry et al., 2000) and different substrate specificities (Gut et al., 2008; Xu et al., 2011) suggest a distinct role of NanB for bacterial pathogenicity and likely the synergism between influenza viruses and *S. pneumoniae*. NanC is rarely expressed (Pettigrew et al., 2006; Walther et al., 2015), and was suggested to act as a regulator for NanA actions (Xu et al., 2011).

Despite the distinct quaternary structures of viral and pneumococcal NAs (viral NA as tetramer and bacterial NAs as monomer), the structure of their active sites show close similarities (von Grafenstein et al., 2015), suggesting that *S. pneumoniae* could be targeted by known NAIs. However, the viral NAI zanamivir does not bind efficiently to the active site of NanA and NanB because some residues involved in key interactions (e.g. Glu119 and Glu227 in the viral NA: N2 numbering) are not conserved in *S. pneumoniae* NAs (Gut

et al., 2011). In contrast, oseltamivir competitively inhibits NanA *in vitro* (Gut et al., 2011; Walther et al., 2015) and also reduces *S. pneumoniae*-induced complications in mice co-infected with influenza virus (McCullers, 2004; Tanaka et al., 2014). Two natural compounds, katsumadain A and the isoprenylated flavone artocarpin, were also described as inhibitors of *S. pneumoniae* NAs (Richter et al., 2015; Walther et al., 2015).

The aim of the present study was to determine the impact of NanB on influenza virus replication in comparison to NanA. Therefore A549 (human lung carcinoma) cell-based models with the A(H1N1)pdm09 isolate A/Jena/8178/09 (Jena/8178) and recombinant NanA or NanB (rNanA or rNanB) were established. This cellular system was used to evaluate the effect of these *S. pneumoniae* NAs on the availability of virus receptors, the virus spread as well as yield, and the efficacy of NAIs on influenza virus replication in the absence or presence of rNanA and rNanB.

RESULTS

rNanA and rNanB Act in Fluorescence (FL)- and Hemagglutination (HA)-based Assay

First rNanA and rNanB activities were compared in FL buffer at pH 6.5 with 60 µM of the substrate MUNANA after two hours of incubation at 37°C. rNanB activity was also measured at pH 5.0. The velocity of rNanB (dilution 1:1,000) was 0.03 ± 0.00 µM/min and 0.94 ± 0.23 µM/min of 4-MU at pH 5 and pH 6.5, respectively. For comparison, 1:1,000 diluted rNanA released 1.63 ± 0.32 µM/min of 4-MU at pH 6.5. Dilutions of 1:10,000 revealed a release of 0.21 ± 0.09 and 0.07 ± 0.04 µM µM/min of 4-MU for rNanA and rNanB, respectively.

Based on these results, the affinity of both enzymes (enzyme dilution 1:10,000) to the substrate MUNANA was determined by measuring their K_m value at pH 6.5. The lower K_m

value of rNanA (39.70 ± 5.81 µM) compared to the K_m value of rNanB (684.03 ± 162.76 µM) indicates a 17-times stronger affinity of rNanA to MUNANA under these assay conditions (Figure 1). The activities of both *S. pneumoniae* NAs were also tested in an HA assay (Richter et al., 2015). Dilutions up to 1:1,200,000 rNanA and 1:250,000 rNanB resulted in hemagglutination of human erythrocytes incubated with peanut lectin, confirming NA activity (results not shown).

NanA and NanB Diminish Virus Receptor Availability

Sialic acids linked via α2-3 or α2-6 to galactose (SAα2-3Gal and SAα2-6Gal) function as receptors for influenza viruses and facilitate their attachment to host cells, the initial step in the viral replication cycle (Nicholls et al., 2013). To study the influence of NanA and NanB on virus receptor expression, SAα2-3Gal and SAα2-6Gal were detected by immunohistochemical staining with the lectins MAA and SNA, respectively. Both influenza A virus receptors were expressed on A549 cells (Figure 2A and B), which is consistent with previously published data (Kumari et al., 2007). SAα2-3Gal and SAα2-6Gal were strongly reduced by NanA at 1:100 and 1:1,000 dilutions compared to the untreated cell control (Figure 2A). A decrease of SAα2-3 was induced by 1:100 and 1:1,000 diluted rNanB whereas no changes in SAα2-6 levels were observed (Figure 2B). No differences between cells treated with 1:10,000 or 1:100,000 diluted NanA and NanB and the virus control were observed. Similar effects were observed in MDCK cells (results not shown).

Distinct Effects of NanA and NanB on Virus Replication

Jena/8178 replicates well in A549 cells (Figure S1). The effect of *S. pneumoniae* NAs on Jena/8178 replication was evaluated in A549 cells by comparing virus spread and yield in the absence and presence of rNanA and rNanB. Therefore, different tenfold dilutions of

rNanA or rNanB were added to virus-infected lung epithelial cells. Forty-eight hours after infection virus spread was analyzed by comparing the number of viral nucleoprotein-positive cells using immunohistochemical staining. In parallel, virus yield in the supernatant was determined by plaque assay.

An increased virus spread was observed when rNanA dilutions of 1:10,000 and 1:100,000 or rNanB dilutions of 1:1,000 up to 1:100,000 (Figure 3A and B) were added. In contrast, dilutions of 1:100 and 1:1,000 of rNanA as well as 1:100 of rNanB hampered the spread of the virus.

In agreement with these results, rNanA dilutions of 1:10,000 and 1:100,000, and rNanB dilutions between 1:1,000 and 1:100,000 increased virus yield (Table 2). On the contrary, rNanA dilutions of 1:100 and 1:1,000 and rNanB dilution of 1:100 reduced the virus titer. The 1:1,000,000 dilutions of both NAs had no effect on virus growth.

The observed, reduced viral spread and yield suggest that high amounts of pneumococcal NAs remove the viral receptors and hinder viral infection. Hence a dilution of 1:10,000 of both pneumococcal NAs supported Jena/8178 replication without affecting SAα2-3Gal and SAα2-6Gal expression in A549 cells. Therefore this dilution was selected to study the effect of NAIs on Jena/8178 spread and yield in the presence and absence of rNanA or rNanB.

NanA and NanB Differ in NAI Susceptibility

Recently we described an HA assay with highly robust readout when we were comparing the activity of NAIs against viral and bacterial NAs (Richter et al., 2015). Especially for the identification of new NAIs this test is more suitable because no artificial substrate is used and inherent color or self-fluorescence of test compounds does not interfere with the assay readout. In the HA assay oseltamivir, DANA, katsumadain A, and artocarpin but, not

zanamivir inhibited rNanA (Table 2). Compared to rNanA, rNanB was ten times less sensitive to oseltamivir. Zanamivir and DANA were inactive. Katsumadain A and artocarpin inhibited both NAs with similar efficiency. rNanB was not susceptible to artocarpin in the FL assay, in contrast to rNanA (Table 2). Apart from this discrepancy, FL assay data confirm the HA assay results. Due to the self-fluorescence of katsumadain A (Richter et al., 2015), the compound could not be tested in the FL assay.

NAIs Help to Overcome the Lethal Synergism of Influenza Viruses and *S. pneumoniae*

The NA of Jena/8178 has been proved to be NAI susceptible (Table S1). Moreover, the tested NAIs reduced in a dose-dependent manner Jena/8178 spread and yield in the absence of pneumococcal NAs in A549 cells (Figure 4, Table S2). Strongest activities were detected for the virus-specific drugs oseltamivir and zanamivir.

The antiviral effect of zanamivir was abolished in the presence of rNanA (Figure 4B and D, Table S2). In contrast, viral yield was inhibited by oseltamivir (1 µM), DANA, katsumadain A, and artocarpin as without NanA. The antiviral activity of the low oseltamivir concentration of 0.1 µM was also hampered by rNanA (Table S2).

All NAIs exerted inhibitory activity in the presence of rNanB and reduced spread as well as yield (Figure 4C and D, Table S2). In conclusion, the presence of rNanB did not affect NAI efficacy against the A(H1N1)pdm09 isolate Jena/8178.

DISCUSSION

The co-pathogenesis of influenza virus and *S. pneumoniae* contributes to high morbidity and mortality of flu epidemics and pandemics (McCullers, 2014). The knowledge of the underlying mechanisms is important for improving treatment measures and helping to prevent this lethal synergism. Here, we demonstrate the crucial role of *S. pneumoniae*

NanA and NanB for influenza virus replication as well as NAI susceptibility. NAIs acting against viral and bacterial NAs were shown to overcome the pneumococcal NA-based synergism in a newly established *in vitro* model. Hence, both bacterial NAs are druggable targets of high importance due to their functional similarity with viral NA.

The role of NanA and NanB in the lethal synergism of *S. pneumoniae* and the influenza virus has not been demonstrated so far. We showed that high amounts of rNanA and rNanB cut off sialic acids from A549 cell surfaces that are used as viral receptors. Jena/8178 was unable to spread and virus yield was diminished. A(H1N1)pdm09 isolates preferentially attach to SA2-6gal in addition to SA2-3gal (Childs et al., 2009; Seidel et al., 2014; Stevens et al., 2006). Therefore, high amounts of rNanA or rNanB, removing SA2-6gal and SA2-3gal or SAα2-3gal, respectively, hamper or even block viral spread to neighboring epithelial lung cells. These results help to explain why synergism only occurs when the viral infection precedes bacterial exposure and no effect – or even protection – occurs when the order of challenge is reversed, reviewed in (McCullers, 2014).

When rNanA or rNanB dilutions were used that do not significantly cleave the sialic acids used as viral receptors from cell surfaces, an opposed effect was observed in A549 cells infected with Jena/8178. The addition of rNanA as well as of rNanB was effective in supporting the viral NA function for viral release. The promotion of A(H1N1)pdm09 strain replication by both *S. pneumoniae* NAs was clearly demonstrated using this cell system. We hypothesize that both pneumococcal NAs are able to contribute to the increase of virus titers observed during bacterial superinfection in which bacterial challenge follows the viral infection *in vivo* (Chockalingam et al., 2012; Smith et al., 2013). The effect will depend on the receptor specificity of a given virus.

The distinct roles of NanA and NanB in influenza virus and *S. pneumoniae* in co-pathogenesis are apparent from the data of inhibition assays with NAIs. As published

recently for NanA (Nishikawa et al., 2012; Richter et al., 2015; Walther et al., 2015) and confirmed by the enzyme inhibition assays in the present study, zanamivir is unable to inhibit the bacterial NAs activity at 100 µM. When co-incubating the virus, zanamivir and rNanA, the function of the blocked viral NA is replaced by rNanA and virus replication occurs. The same effect was observed for the low oseltamivir concentration (0.1 µM) that is ineffective against rNanA (Nishikawa et al., 2012; Richter et al., 2015). In contrast, virus yield and spread remain reduced in the presence of NanB, leading to the conclusion that NanB cannot replace Jena/8178 NA as does NanA. Unlike for NanA, which can cleave both SA linkages, NanB has limited activity in cleaving only SAα2-6gal. For this reason, NanB can support but does not completely replace the cleavage function of Jena/8178 NA necessary for effective viral release and spread. In agreement with the good susceptibility of NanA to oseltamivir, this NAI also inhibits viral spread after addition of NanA. Compared to the inhibition of viral NA this activity is markedly lower because of structural differences within the active site (Gut et al., 2011). We modeled the three NAs in question with oseltamivir and zanamivir bound (Figure 5). These models are in agreement with the collected *in vitro* data and are able to explain the observed bioactivity. It was found that steric clashes are the primary reason for inactivity on one or several of these structurally related enzymes (Walther et al., 2015).

In contrast to oseltamivir, katsumadain A and artocarpin were similar effective against NanA and NanB in the HA assay and inhibited also the viral NA, which underlines our previous finding about an extended interaction of these natural compounds in the NA binding site (Grienke et al., 2010; Kirchmair et al., 2011; Richter et al., 2015; Walther et al., 2015). They also affected viral replication. Moreover, artocarpin exerted antibacterial activity. Therefore, we suggest that its structure could provide the base for the search of compounds with stronger activity against influenza viruses and *S. pneumoniae.*

Taken together our findings suggest distinct roles for NanA and NanB in the lethal synergism of A(H1N1)pdm09 strains and *S. pneumoniae* as well as for influenza treatment with NAIs. NanB plays a minor part according to our test system. NanB has the potential to support spread of A(H1N1)pdm09 strains but not if the viral NA is blocked by NAIs. It should be considered that this effect might depend on the specific virus strain due to differences in the use of SAα2-6Gal and SAα2-3Gal as virus receptor (Kumari et al., 2007). For example, the impact of NanB on avian or avian-like swine viruses that preferentially bind to SAα2-3Gal might be much stronger (Bauer et al., 2012; Nicholls et al., 2013).

In conclusion, NanA and NanB promote virus replication. The effect of viral NAIs that are unable to inhibit the pneumococcal NanA can be diminished by functional replacement. In consequence, only NAIs targeting viral and pneumococcal NanA help to prevent the lethal synergism.

EXPERIMENTAL PROCEDURE

Compounds

Oseltamivir carboxylate (GS4071) and zanamivir (GG167) were kindly provided by GlaxoSmithKline (Uxbridge, UK) and F. Hoffmann-La Roche AG (Basel, Switzerland), respectively. N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) was purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany). Artocarpin (Quality Phytochemicals LLC, East Brunswick, NJ, USA, (Kirchmair et al., 2011)) and katsumadain A, previously isolated from the seeds of *Alpinia katsumadai* Hayata (Grienke et al., 2010) were dissolved in DMSO. The diluent of all other compounds was aqua bidest. Compound stocks (10 mM) were stored at 4 °C, with the exception of DANA, which was stored at -20 °C, until use.

Cells, media, and cultivation

Human lung carcinoma cells (A549; Institute of Molecular Virology, University of Münster, Germany) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Lonza Group Ltd, Basel, Switzerland) supplemented with 10% foetal calve serum (PAA Laboratories GmbH, Cölbe, Germany). Madin-Darby canine kidney (MDCK) cells (Friedrich-Loeffler Institute, Riems, Germany) were grown in Eagle's minimum essential medium (EMEM; Lonza Group Ltd) supplemented with 10% fetal bovine serum (Sigma-Aldrich GmbH), and 2 mM L-glutamine (Lonza Group Ltd), 10% non-essential amino acids (Lonza Group Ltd), 1mM sodium pyruvate (Lonza Group Ltd). Cells were cultivated at 37°C with 5% CO_2.

Serum-free DMEM (A549 cells) or EMEM (MDCK cells) medium containing 0.25 µg/mL (A549 cells) or 2 µg/ml (MDCK cells) trypsin (Sigma-Aldrich GmbH) and 1.3% sodium bicarbonate (Lonza Group Ltd) was used for *in vitro* studies (test medium) with virus.

Virus propagation

Stock of the A(H1N1)pdm09 isolate A/Jena/8178/09 (Jena/8178) was prepared in serum-free EMEM medium containing 2 mM L-glutamine, 2 µg/mL trypsin, and 0.1% sodium bicarbonate in mycoplasma-free MDCK cells. Aliquots (4.28×10^7 $TCID_{50}$/50 µl) were stored at -80 °C until use.

Expression of r NanA and rNanB

The expression and purification of *nanA* of *S. pneumoniae* DSM20566 has been described previously (Walther et al., 2015). The *nanB* from the same strain was amplified with the primers: nanB_NcoI_fw (5'-GGAAAACCATGGATAAAAGAGG-3') and nanB_XhoI_rv (5'-TTCTCTCGAGTTTTGTTAAATC-3'). After the double-digestion with *Nco*I and *Xho*I, the ~2.1 kb PCR product was cloned into *E. coli* expression vector pET-28a. The

obtained plasmid pTNB20566 encodes a C-terminal 6xHis-tagged NanB in size of 699 aa residues. Expression and purification of NanB used in this study was done as described previously for NanA (Walther et al., 2015).

Analysis of pneumococcal NA activity

Activity of rNanA and rNanB was compared with FL assay as published (Richter et al., 2015). Tenfold dilutions (1:1,000 to 1:100,000) of NAs in FL buffer (32.5 mM MES, 4 mM calcium chloride, pH 6.5 for rNanA, pH 5 and pH 6.5 for rNanB) were incubated with 60 µM substrate 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate (MUNANA, Sigma-Aldrich GmbH) at 37°C for 20 minutes. After 2 hours, 150 µl stop solution (0.1 M glycine (Sigma-Aldrich GmbH), 25% absolute ethanol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), pH 10.7) was added, relative FL units (RFU) were read (excitation wavelength of 355 nm and emission wavelength of 460 nm) using the microplate reader FLUOstar Omega (BMG Labtech GmbH, Ortenberg, Germany) and converted to 4-MU concentration (Sigma-Aldrich GmbH) according to the 4-MU standard curve (Figure S2). Velocity (µM/min) and K_m value (µM) were determined for rNanA and rNanB. Therefore, 30 µl of MUNANA (eight different concentrations from 400 µM to 3.13 µM) were incubated with 10 µl buffer and 10 µl 1:10,000 diluted NA in FL buffer (pH 6.5). After 5 minutes reaction was stopped, RFU were measured and converted to 4-MU concentration according to the 4-MU standard curve. The EnzymeKinetics Module of SigmaPlot 12.0 (Systat Software, San Jose, CA) was applied to fit the data to the Michaelis-Menten equation using nonlinear regression.

NA activity of both NAs was further confirmed in hemagglutination-based assays (HA assay) with human erythrocytes described recently (Richter et al., 2015; Walther et al., 2015).

302 NA inhibition assays

303 The inhibitory effect of NAIs on rNanA and rNanB was evaluated with the FL assay
304 (rNanA and rNanB diluted 1:10,000 in FL buffer, pH 6.5) and hemagglutination-based NA
305 inhibition assays (HA assay) as published recently (Richter et al., 2015) with slight
306 modification of the HA assay. For stability reasons, rNanA and rNanB were diluted
307 1:5,000 in PBS containing 100 µg/ml BSA and stored at -20°C until use. In addition, the
308 incubation period of the recombinant proteins with the human erythrocytes of 4 h was
309 reduced to one hour. At least three individual experiments were performed. NAI
310 susceptibility of Jena/8178 NA was proved with FL assay.

311 Co-incubation model of influenza virus with rNanA or rNanB

312 Three-day-old confluent A549 cells seeded (2.0 cells/ml) on 24-well tissue culture plates
313 (Greiner Bio-One GmbH, Frickenhausen, Germany) or 8-well plastic slides (Thermo
314 Scientific™ Nunc™, Schwerte, Germany) were infected with Jena/8178 (MOI of
315 0.1 $TCID_{50}$/cell) for one hour at 37°C in 5% CO_2 enriched atmosphere, respectively. A cell
316 control (uninfected cells) was included in duplicate. After three washing steps with test
317 medium, tenfold diluted rNanA or rNanB (1:100 to 1:1,000,000) were added in a volume
318 of 500 µL or 400 µL medium per well for 24-well plate or for 8-well slide, respectively.
319 No recombinant NA was added to the two virus control and cell control wells. After 48
320 hours at 37°C the supernatant of the 24-well plate was taken and stored at -20°C until
321 plaque titer determination (virus yield analysis). In parallel, cells on the slide were fixed
322 with ice-cold methanol for 20 min and stored after washing with phosphate buffered saline
323 (PBS) in PBS at 4°C until immunohistochemical staining (virus spread analysis).

324 Effect of NAIs in the co-incubation model of influenza virus with rNanA or rNanB

325 The inhibitory effect of the NAI on Jena/8178 (inhibition of virus yield and spread) was

326 determined in absence and presence of rNanA or rNanB in A549 cells. NA dilutions were
327 used that (i) did not reduce the amount of sialic acid (SA) α2-3Galactose (Gal) and SAα2-
328 6Gal but, (ii) significantly increased virus yield and spread.

329 Analysis of virus spread by immunohistochemical staining

330 Viral antigen expression was analyzed by detection of viral nucleoprotein with a
331 monoclonal antibody (Acris GmbH, Hiddenhausen, Germany) in 4 h- as well as 48 h-
332 infected, methanol-fixed cells on the 8-well plastic slide by immunohistochemical staining
333 according to the manufacturers protocol (DAKO, Hamburg, Germany) as described
334 elsewhere (Bauer et al., 2007). Slides were microscopically analyzed with Zeiss Axioskop
335 2 microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany).

336 Determination of virus yield

337 To determine the virus yield in the supernatant from co-incubation experiments, standard
338 plaque assay was conducted on 2- to 3-day-old confluent MDCK cells grown in 12-well
339 tissue culture plates (Greiner Bio-One GmbH). Briefly, after virus adsorption at 37°C for
340 one hour, inoculum was replaced by 1 mL test medium containing also 0.4% agarose and
341 50 mM magnesium chloride (VEB Laborchemikalien, Apolda, Germany). The cells were
342 incubated at 37°C for 48 h until plaques appeared and then fixed and stained with a
343 solution of 0.4% crystal violet in a mixture of formalin (3% v/v) and ethanol (1.67% v/v)
344 in water overnight. Plaques were counted over a light box after removal of the agar
345 overlay. The average plaque count from virus control was set to 100% and percentage of
346 plaque reduction by NAIs was calculated.

347 Detection of sialic acid (SA) α2-3Galactose (Gal) and SAα2-6Gal by lectin-binding assay
348 on A549 cells

349 The effect of pneumococcal NAs on cell surface sialic acids was investigated by

immunohistochemical staining with DIG glycan differentiation kit (F. Hoffmann-La Roche AG) as reported (Seidel et al., 2014). Briefly, confluent A549 or MDCK cells grown on 8- or 16-well slides were incubated for 48 hours at 37°C with 1:100 to 1:100,000 diluted NanA or NanB. After methanol fixation for 20 minutes slides were washed with PBS and lectin staining with DIG-labeled *Sambucus nigra* agglutinin (SNA; 1 µg/mL) or *Maackia amurensis* agglutinin (MAA; 5 µg/mL) followed according to the manufacturer instructions. SNA recognizes SAα2-6Gal and MAA SAα2-3Gal. For the red staining chromogenic solution of the DAKO REAL Detection System APAAP, Mouse was applied (DAKO). Slides were microscopically analyzed.

Molecular modeling

Homology models of Jena/8178 and NanA and NanB of *S. pneumoniae* DSM20566 were generated with Swiss-Model (Biasini et al., 2014) using PDB 4b7q, 2vvz and 2jkb as templates, respectively. Oseltamivir and zanamivir were extracted from co-crystallized structures (2hu0, 4b7q and 2htr) aligned with Maestro (Release, 2014) and inserted into the homology models. Residue numbering of the template structures was preserved. Graphics were rendered using PyMol (Schrodinger, 2013).

Statistical analysis

Mean and standard deviation were analyzed using Microsoft Excel 2010. Significant differences in the co-incubation assay were calculated in SPSS Statistics Version 22 with non-parametric Wilcoxon–Mann–Whitney test.

AUTHOR CONTRIBUTION

SHP and SM designed research;

WE, XZ, RM, KJ, GU, KA performed research;

PW, RJM, SA, SM contributed reagents/analytic tools;

WE, XZ, RM, KJ, KA, GU, RJM, SM analyzed data; and

WE, XZ, RM, KJ, KA, GU, RJM, SA, SHP, PW, SM wrote the paper.

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FIGURES

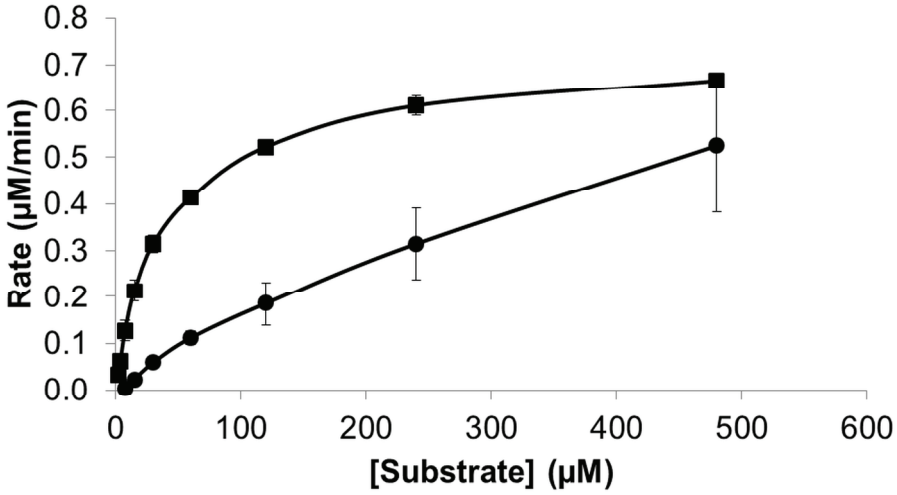

Figure 1. Cleavage Activitiy of NanA and NanB. Michaelis Menten curves of NanA (squares) and NanB (dots) at pH 6.5. Both NAs were diluted 1:10,000 and incubated for 5 minutes with different substrate (MUNANA) concentrations. Kinetics was conducted three times. Mean and standard deviations were used to generate the graph.

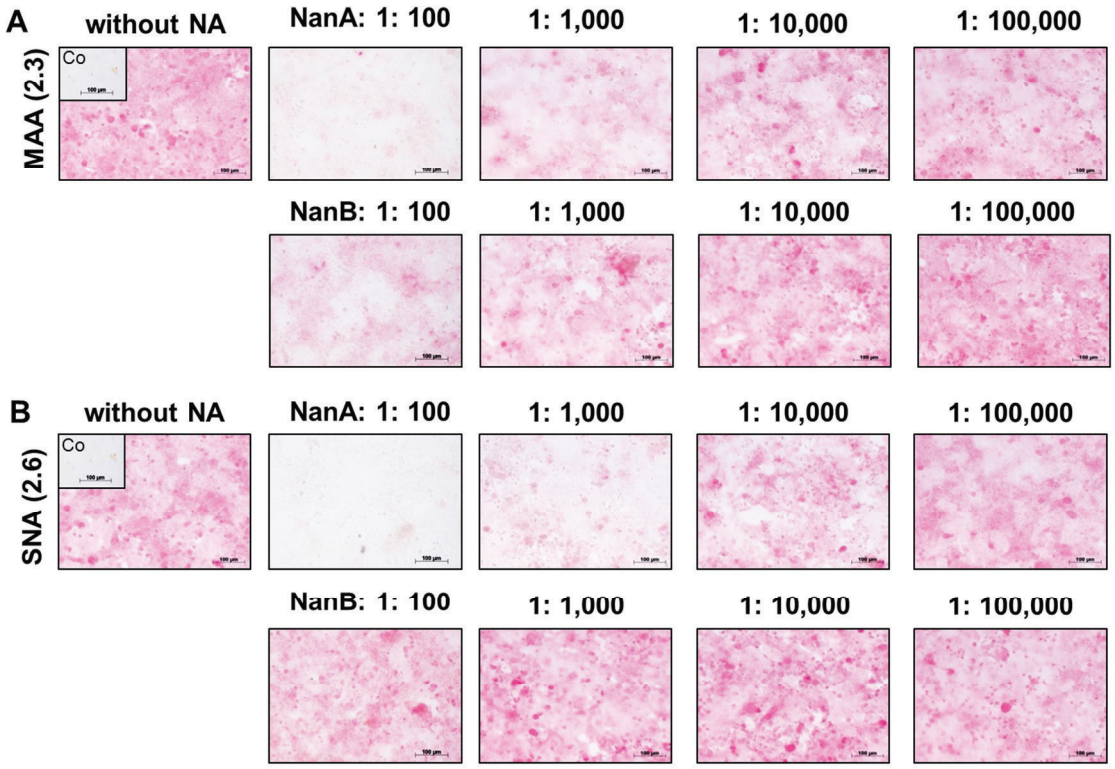

Figure 2. Influence of NanA and B on Expression of Sialic Acids on the Surface of A549 Cells. Cells were treated with different neuraminidase dilutions for 48 h. The lectins MAA and SNA were used to detect SAα2-3Gal and SAα2-6Gal, respectively, by immunohistochemical staining. Control (Co) were stained without using lectins.

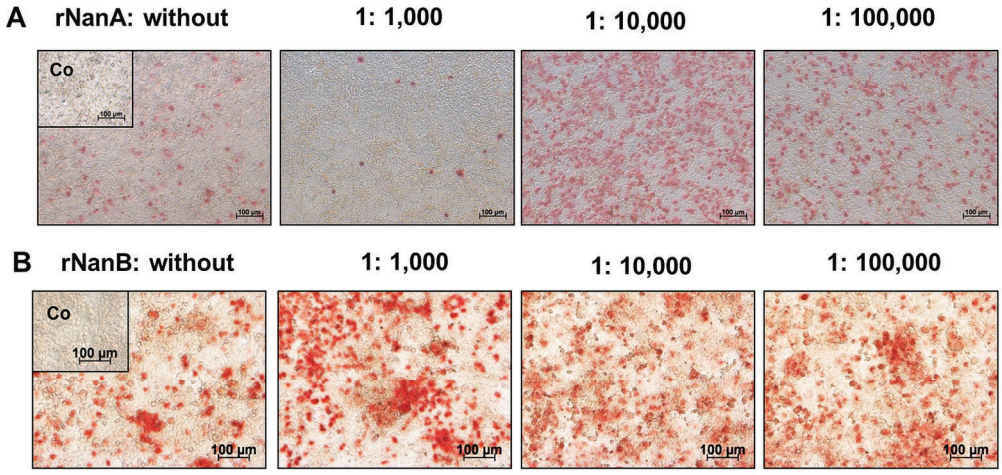

Figure 3. Spread of A(H1N1)pdm09 Strain A/Jena/8178/09 (Jena/8178) in Absence and Presence of Different Dilutions of Recombinant NanA and NanB. The effect of NanA (A) and NanB (B) on virus spread in A549 cells was analyzed by immunohistochemical staining of viral nucleoprotein (shown in red) 48 h after infection with Jena/8178 at MOI of 0.1 TCID50/cell.

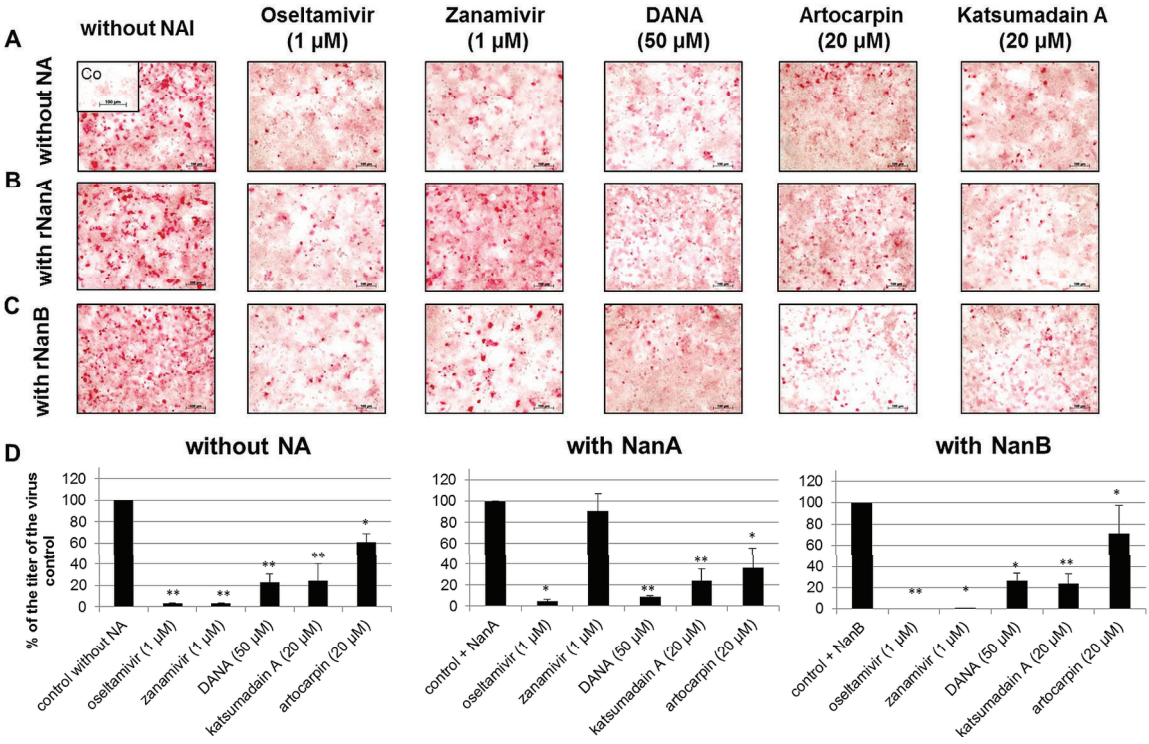

Figure 4. Inhibition of Replication of A(H1N1)pdm09 strain A/Jena/8178/09 (Jena/8178) by Neuraminidase Inhibitors (NAIs). A549 cells infected with Jena/8178 (MOI of 0.1 TCID50/cells) were treated with oseltamivir, zanamivir, DANA, artocarpin, and katsumadain A in absence of pneumococcal NAs (A), presence of rNanA (B) or presence of rNanB (C). Virus-infected cells were detected by immunohistochemical staining of viral nucleoprotein (shown in red) 48 h after infection to visualize the inhibitor effect on virus spread. To analyse the effect of NAIs on virus yield (D), virus titers in pfu/ml were determined with plaque assay 48 h after infection. Virus control titer was set to 100% and inhibition of the control titer by NAIs in % was calculated. Experiments were performed at least three times, and one representative assay is exemplarily shown. Significant values were calculated with non-parametric Wilcoxon–Mann–Whitney test (* $p<0.5$, ** $p<0.1$).

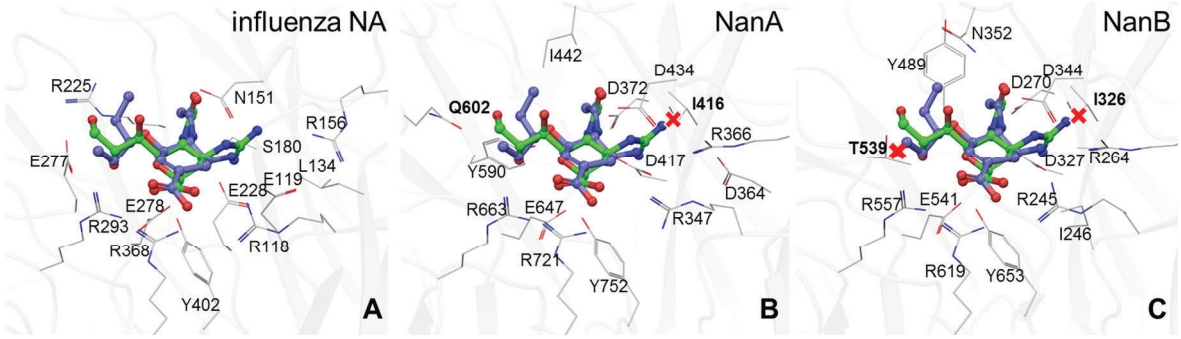

Figure 5. Computational Models of the Binding of Oseltamivir (carbons blue) and Zanamivir (carbons green) to A/Jena/8178/09 (A), NanA (B) and NanB (C). While both inhibitors fit well to the viral NA (A), a steric clash (marked by a red X) of zanamivir with I416 in NanA (B) and the equivalent in NanB, I326, and T539 (C) is apparent. This is the likely cause for the observed inactivity of zanamivir on both bacterial NAs.

TABLES

Table 1. Effect of Recombinant NanA (rNanA) and NanB (rNanB) on Virus Yield.

dilution of NAs	percentage of virus yield in the presence of	
	rNanA [a]	rNanB [a]
1:100	2.8 ± 4.6	15.5 ± 4.5
1:1,000	12.2 ± 5.7	204.8 ± 77.5
1:10,000	234.4 ± 70.5	202.5 ± 107.3
1:100,000	171.7 ± 37.1	186.1 ± 34.5
1:1,000,000	103.2 ± 31.7	114.8 ± 47.4

[a] The plaque titer of influenzavirus A/Jena/8178/09 reached in A549 cells (48 h after infection) was determined in MDCK cells. Plaque titer without pneumococcal neuraminidases was set to 100% virus yield and used to calculate the percentage of virus yield in presence of rNan A and rNanB. Mean and standard deviations of at least two assays with two replicates are shown.

Table 2. Inhibition of Recombinant NanA (rNanA) and NanB (rNanB) by Neuraminidase Inhibitors (NAIs).

NAI	Inhibitory concentration of NAIs in µM against			
	rNanA		rNanB	
	FL assay [a]	HA assay [b]	FL assay [a]	HA assay [b]
oseltamivir	2.9 ± 1.0	3.2 ± 0.0	76.8 ± 27.4	31.6 ± 0.0
zanamivir	not active	not active	not active	not active
DANA	17.7 ± 2.0	100.0 ± 0.0	not active	not active
katsumadain A	not evaluable [c]	3.2 ± 0.0	not evaluable [c]	5.4 ± 4.0
artocarpin	10.0 ± 6.3	10.0 ± 0.0	not active	10.0 ± 0.0

[a] Mean 50% inhibitory concentration (IC_{50}) and standard deviation determined in FL assay in at least three independent assays. The maximum tested concentration was 100 µM

[b] Mean minimal inhibitory concentration (MIC) and standard deviation determined in HA assay in at least three independent assays. The maximum tested concentration was 100 µM

[c] not evaluable due to self-fluorescence of the compound (Richter et al., 2015)

SUPPLEMENTAL INFORMATION

Supplemental information includes two tables as well as two figures and two tables can be found with this article online at XXX.

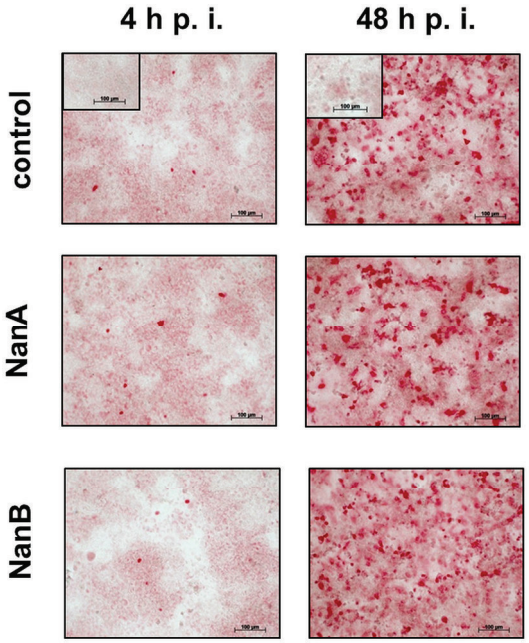

Figure S1. Confirmation of A(H1N1)pdm09 Strain A/Jena/8178/09 (Jena/8178) Replication in A549 Cells. Jena/8178 was propagated in absence and presence of different dilutions of recombinant NanA and NanB. Viral replication was analyzed by immunohistochemical staining of viral nucleoprotein (shown in red) after infection with Jena/8178 at MOI of 0.1 $TCID_{50}$/cell.

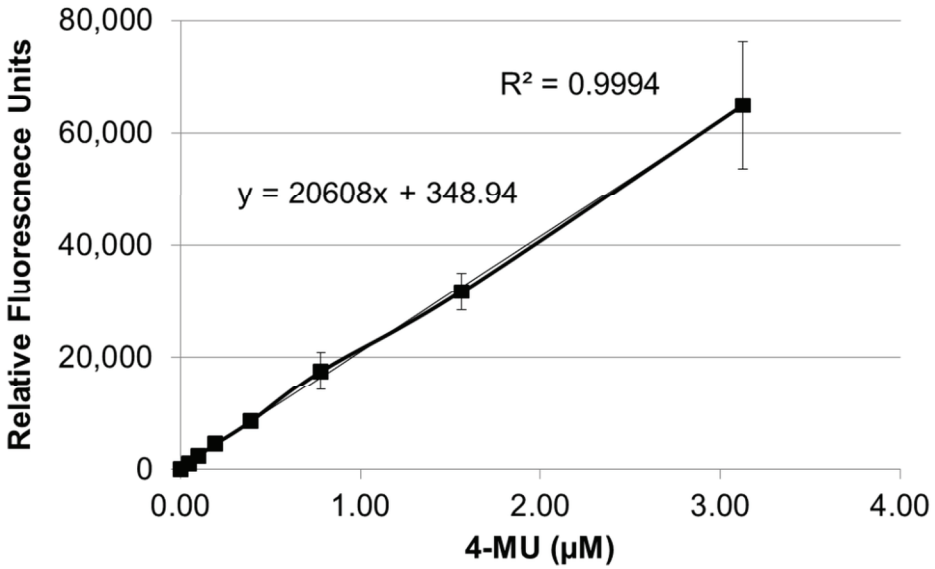

Figure S2. Standard Curve of the Fluorescence Product 4-MU.

Table S1. Inhibition of the Influenza Virus A/Jena/8178/09 Neuraminidase by Neuraminidase Inhibitors (NAIs).

NAI	Inhibitory concentration of NAIs in µM FL assay [a]
oseltamivir	0.003 ± 0.001
zanamivir	0.005 ± 0.000
DANA	3.6 ± 0.4
katsumadain A	not evaluable [b]
artocarpin	41.8 ± 10.7

[a] Mean 50% inhibitory concentration (IC_{50}) and standard deviation determined in FL assay in at least three independent assays. The maximum tested concentration was 100 µM.
[b] not evaluable due to self-fluorescence of the compound

Table S2. Effect of Recombinant NanA (rNanA) and NanB (rNanB) on Inhibition of Virus Yield by Neuraminidase Inhibitors (NAIs).

NAI	concentration (µM)	percentage of virus yield after NAI treatment [a]		
		without NA	with rNanA	with rNanB
oseltamivir	1	1.5 ± 1.1	3.2 ± 1.4	0.5 ± 0.2
	0.1	16.7 ± 11.0	67.8 ± 24.8	4.5 ± 0.2
zanamivir	1	1.4 ± 1.0	86.5 ± 46.2	0.7 ± 0.3
	0.1	4.4 ± 1.5	109.0 ± 35.5	6.2 ± 2.9
DANA	50	34.8 ± 17.4	13.5 ± 8.3	27.4 ± 1.0
	10	78.9 ± 36.1	81.1 ± 27.9	not tested
katsumadain A	20	29.0 ± 15.1	28.1 ± 13.7	21.0 ± 4.1
	10	54.9 ± 25.7	67.3 ± 6.2	not tested
artocarpin	20	84.8 ± 15.7	44.3 ± 11.0	77.1 ± 37.8
	10	70.2 ± 10.5	67.3 ± 25.5	not tested

[a] The plaque titer of untreated control (influenzavirus A/Jena/8178/09) reached in A549 cells (48 h after infection) in the absence and presence of rNanA or rNanB was set as 100% virus yield. It was used to calculate the percentage of virus yield after NAI treatment. Mean and standard deviations were calculated of at least three assays with two replicates.

3 Identifizierung neuer NA-Inhibitoren (Azo-Verbindungen)

Zum Abschluss der Arbeit soll anhand von Azo-Verbindungen gezeigt werden, wie mit Hilfe von Computermodellierungen erfolgreich Leitstrukturen identifiziert werden können. Dazu werden die Wirkungen der neuen Substanzen gegen Pneumokokken-NA sowie Influenzaviren und Pneumokokken in enzymbasierten Testsystemen sowie in antiviralen und antibakteriellen Hemmtests untersucht. Die Azo-Verbindungen, die in allen Testsystemen gegen beide Pathogene hohe Hemmwirkungen erzielen, werden schließlich im Koinkubationsmodell auf ihren inhibitorischen Effekt geprüft.

3.1 Die Suche nach neuen NA-Inhibitoren

In den letzten Jahren hat die Suche nach neuen resistenz-brechenden NAI deutlich zugenommen. Dabei wurden die zu testenden Substanzen in einem ersten Schritt mittels virtuellen Screenings identifiziert (Grienke *et al.*, 2012; Hsu *et al.*, 2013). In der vorliegenden Dissertation konnte eine Serie von Substanzen (n = 18) charakterisiert werden, die wie andere, bereits publizierte Anti-Influenzavirus-Substanzen ein Chromophor und eine Säuregruppe enthalten (Amaro *et al.*, 2009; Hsu *et al.*, 2013). Allerdings wurde keine der Substanzen bisher als Medikament zugelassen. Die vorliegende Grundstruktur zeigt ein Molekül bestehend aus einer Naphtalingruppe, zwei Benzolringen mit zwei Azogruppen, einer Sulfonsäuregruppe sowie einer Hydroxylgruppe (Abbildung 3). Die einzelnen Substanzen der Serie der Azo-Verbindungen enthalten bis zu zwei Naphtalingruppen, zwei Benzolringe, drei Azogruppen, vier Sulfonsäuregruppen und einer Nitrogruppe. Die Strukturformeln aller getesteten Substanzen befinden sich im Anhang (Seite 105).

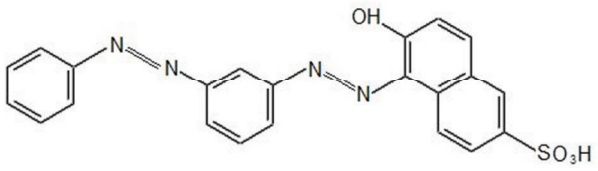

Abbildung 3: Grundstruktur Azo-Verbindung. Substanz 65545 ist aus einer Naphtalingruppe, zwei Benzolringen, zwei Azogruppen, einer Hydroxylgruppe sowie einer Sulfonsäuregruppe aufgebaut.

3.2 Antivirale und antibakterielle Wirkungen

Zunächst wurden alle 18 Substanzen auf ihre Verträglichkeit in Hundenierenzellen (MDCK-Zellen) getestet, wobei sich alle untersuchten Azo-Verbindungen bis zu einer Konzentration von 100 µM als gut verträglich erwiesen (Ergebnisse nicht dargestellt). Et-

wa 10 µM der Substanzen 73416, 65557 und 65826 hemmten den virusinduzierten zytopathischen Effekt zu 50 % (IC_{50}) (Tabelle 1). Die IC_{50} von Substanz 65551 befand sich sogar bei 3 µM. Die genannten Verbindungen wirkten ebenso gut auf das planktonische Wachstum der Bakterien, jedoch deutlich schlechter auf die Biofilmbildung.

Die Substanzen wurden zudem im FL-Test auf ihre Wirkung bezüglich der Pneumokokken-NA untersucht. Wie in Publikation 2 kam das Pneumokokken-Präzipitat von Referenzstamm DSM20566 zum Einsatz. Die Substanzen 65847, 45601, 73416 und 65826 hemmten im FL-Test die NA-Aktivität des Pneumokokken-Präzipitats bei einer Konzentration von ca. 2 µM zu 50 %. Im enzymbasierten Hemmtest wirkten die Verbindungen mindestens ebenso gut wie in den biologischen Testsystemen.

Tabelle 1: Antivirale und antibakterielle Aktivitäten der Azo-Verbindungen. Für die Hemmung der Virusreplikation im zytopathischen Effekt-Hemmtest sind die 50 % inhibitorischen Konzentrationen der Substanzen gegen Influenzavirus A/Jena/8178/09 (Jena/8178) angegeben. Die Pneumokokken von Referenzstamm DSM20566 sowie deren Präzipitat wurden in antibakteriellen Untersuchungen bzw. im Fluoreszenz(FL)-Test getestet. Für die Auswertung wurden mindestens drei unabhängige Tests durchgeführt.

	50 % inhibitorische Konzentration (µM)			
	Virusreplikation Jena/8178 [a]	planktonisches Wachstum [b]	Biofilm-bildung [b]	FL-Test [a]
Substanz	**Virus**	**Pneumokokken**		**Pneumokokken-Präzipitat**
65847	20,36 ± 2,51	28,75 ± 13,13	49 %[c]	0,32 ± 0,05
45601	nicht aktiv	45 %[c]	42,23 ± 2,57	0,97 ± 0,30
73416	9,09 ± 1,94	4,59 ± 2,45	47 %[c]	1,85 ± 0,10
45582	nicht aktiv	11,97 ± 6,81	46 %[c]	7,68 ± 0,98
65545	14,42 ± 8,16	20,14 ± 2,24	nicht aktiv	11,62 ± 0,15
65551	3,28 ± 2,19	3,32 ± 0,72	46 %[c]	5,68 ± 3,01
65557	12,10 ± 4,83	11,97 ± 6,81	46 %[c]	4,79 ± 1,31
58049	17,06 ± 8,98	27,59 ± 18,87	nicht aktiv	-[d]
45576	nicht aktiv	nicht aktiv	nicht aktiv	5,74 ± 1,41
65826	10,58 ± 0,71	6,14 ± 0,61	46 %[c]	1,18 ± 0,55
75957	nicht aktiv	nicht aktiv	nicht aktiv	46,79 ± 5,27
75953	nicht aktiv	nicht aktiv	nicht aktiv	10,18 ± 1,35
65553	nicht aktiv	nicht aktiv	nicht aktiv	3,97 ± 0,51
58050	nicht aktiv	nicht aktiv	nicht aktiv	31,96 ± 10,92

45538	nicht aktiv	nicht aktiv	nicht aktiv	23,19 ± 13,12
45549	nicht aktiv	nicht aktiv	nicht aktiv	nicht aktiv
45540	nicht aktiv	nicht aktiv	nicht aktiv	nicht aktiv
45541	nicht aktiv	nicht aktiv	nicht aktiv	nicht aktiv

[a] höchste eingesetzte Konzentration: 100 µM
[b] höchste eingesetzte Konzentration: 50 µM
[c] Hemmung der Pneumokokken bei der maximal eingesetzten Konzentration von 50 µM
[d] nicht auswertbar aufgrund von Eigen-FL (Publikation Richter *et al.* in Vorbereitung)

3.3 Wirkungen im Koinkubationsmodell

Zum Abschluss der Charakterisierung erfolgte die Prüfung der beiden Substanzen 65847 und 73416 im Koinkubationsmodell mit Influenzavirus Jena/8178 und rNanA der Pneumokokken (Abbildung 4). Es stellte sich heraus, dass beide Substanzen in der Lage sind, die virale Replikation in An- und in Abwesenheit von rNanA zu unterdrücken. Die virale Ausbreitung wurde blockiert, was zu einem reduzierten Virusertrag führte.

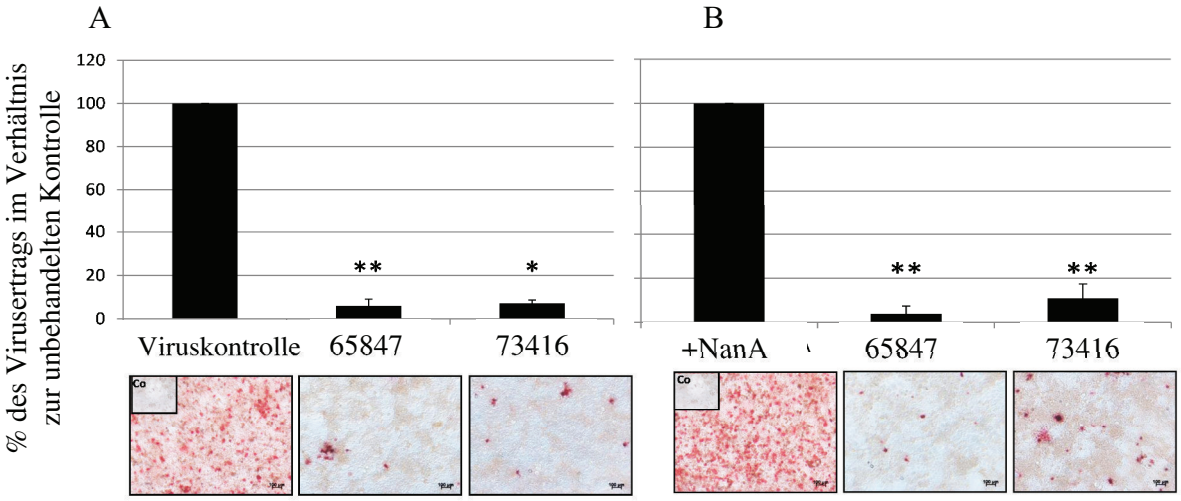

Abbildung 4: Wirkung der Azo-Verbindungen 65847 und 73416 im Koinkubationsmodell. Lungenepithelzellen wurden mit dem pandemischen Influenza-A-Virus A/Jena/8178/09 (MOI 0,1) infiziert. Nach einstündiger Adsorption erfolgte die Zugabe von 10 µM Substanz (A und B) und rNanA in der Verdünnung 1:10.000 (B). Nach 48-stündiger Inkubation wurde der Überstand abgenommen und im Plaquetest titriert. Dargestellt sind die Viruserträge im Verhältnis zur unbehandelten Kontrolle, die auf 100 % skaliert wurde. Parallel wurden die Zellen methanol-fixiert und das virale Nukleoprotein immunohistochemisch angefärbt (rot). In der linken oberen Bildecke ist die nicht-infizierte Zellkontrolle (Co) abgebildet.

4 Diskussion

Influenzaviren und Pneumokokken exprimieren NA als Virulenzfaktoren (Grienke *et al.*, 2012; Manco *et al.*, 2006; Paton *et al.*, 1993). Würde es gelingen, die virale und die bakteriellen NA mit nur einem Wirkstoff zu hemmen, könnten bakterielle Sekundärinfektionen mit hoher Mortalität möglicherweise verhindert werden. Die vorliegende Dissertation zeigt anhand von bekannten viralen NAI und neuen Inhibitoren *in vitro*, dass (a) es möglich ist, NAI mit dualer Wirkung zu identifizieren und (b) diese das Potenzial besitzen, auf die Koinkubation von Influenzavirus mit bakterieller NA einzuwirken.

4.1 NA von *S. pneumoniae* – Ein gutes Wirkstofftarget?

Zu Beginn der Arbeit stellte sich die Frage, ob die bakterielle NA, ebenso wie die virale NA, ein gutes Zielprotein darstellt. Dazu sollte diese als Virulenzfaktor möglichst in jedem Pneumokokkenstamm vorhanden und die Bindetasche hochkonserviert sein. Alle untersuchten klinischen Isolate exprimieren sowohl die NanA als auch zu 96 % die NanB (Publikation 2, Tabelle 2). Die NA-Aktivität wurde für alle Stämme im FL-Test nachgewiesen. Vier Stämme der Stammsammlung produzieren zudem die dritte Pneumokokken-NA, NanC. Ähnliche Verhältnisse finden sich auch in anderen Arbeiten (Pettigrew *et al.*, 2006).

Tabelle 2: Pneumokokkenstämme und -isolate, die in der vorliegenden Dissertation verwendet wurden.

Isolat/Stamm	Herkunft	Serotyp	NanA	NanB	NanC
DSM20566 [P1, P2, P3]	Referenzstamm	1	x	x	-
rNanA [P2, P3]	NanA von DSM20566		x	-	-
rNanB [P3]	NanB von DSM20566		-	x	-
DSM14378 [P2]	Referenzstamm	5	x	x	-
D39 [P2]	Referenzstamm	2	x	x	-
BC2042	Sepsis	-[a]	x	x	-
BC57 [P2]	Sepsis	7F	x	x	-
BC7326 [P2]	Sepsis	22F	x	x	x
CF6852	zystische Fibrose	11A	x	x	x
CF6857	zystische Fibrose	23A	x	x	x
CF6937 [P2]	zystische Fibrose	38	x	-	x
CF8919 [P2]	zystische Fibrose	19A	x	x	-
CF9800	zystische Fibrose	6C	x	x	-

PCD8755	primäre ziliäre Dyskinesie	10A	x	x	-
CJ9400 [P2]	Konjunktivitis	18C	x	x	-
PN8828 [P2]	Pneumonie	6C	x	x	-

[a] zu wenig mikrobiologisches Material bei der Serotypisierung vorhanden
[P1, P2, P3] Pneumokokkenstämme wurden in den Publikationen 1 (P1), 2 (P2) und 3 (P3) verwendet

Die Serotypen wurden von der Arbeitsgruppe von Dr. Mark van der Linden vom Nationalen Referenzzentrum für Streptokokken in Aachen bestimmt. Es zeigte sich, dass zehn der 14 Stämme im Polysaccharid-Impfstoff enthalten sind. Ausnahmen bilden die Isolate CF6857, CF6937, CF9800 und PN8828.

Strukturelle Ähnlichkeit der NA

Es schloss sich die Frage an, ob die strukturelle Ähnlichkeit und die Variabilität der NA von Virus und Bakterien es erlauben, dual-wirksame NAI zu identifizieren. Die primäre Aminosäuresequenz der Influenza-NA stimmt zwar nur zu etwa 10 % mit jener der bakteriellen NA überein (von Grafenstein *et al.*, 2015). Innerhalb der bakteriellen Enzyme liegt die Sequenzidentität aber bei ca. 30 % (Taylor, 1996). In dem berechneten phylogenetischen Stammbaum (Abbildung 5) mit verschiedenen bakteriellen NA und Influenza-NA fällt auf, dass die Pseudaminidase von *Pseudomonas aeruginosa* sich von allen anderen NA am stärksten unterscheidet und als Außengruppe erscheint. Weiterhin wird deutlich, dass sowohl die bakteriellen NA als auch die viralen NA der pandemischen Viren eine Gruppe bilden. Innerhalb der bakteriellen Verzweigung ist die NA von *Vibrio cholerae* und NanH von *C. perfringens* am weitesten von den drei Pneumokokken-NA sowie von NanI und NanJ von *C. perfringens* entfernt. Der Stammbaum wurde bereits in ähnlicher Form publiziert, aber hier noch um NanB und NanC erweitert (Parker *et al.*, 2009). Werden diese beiden NA phylogenetisch betrachtet, zeigt sich, dass sie nebeneinander in der gleichen Verzweigung wie NanA sowie NanI und NanJ von *C. perfringens* stehen. Strukturell weisen sie folglich größere Ähnlichkeiten zueinander auf als im Vergleich zu NanA.

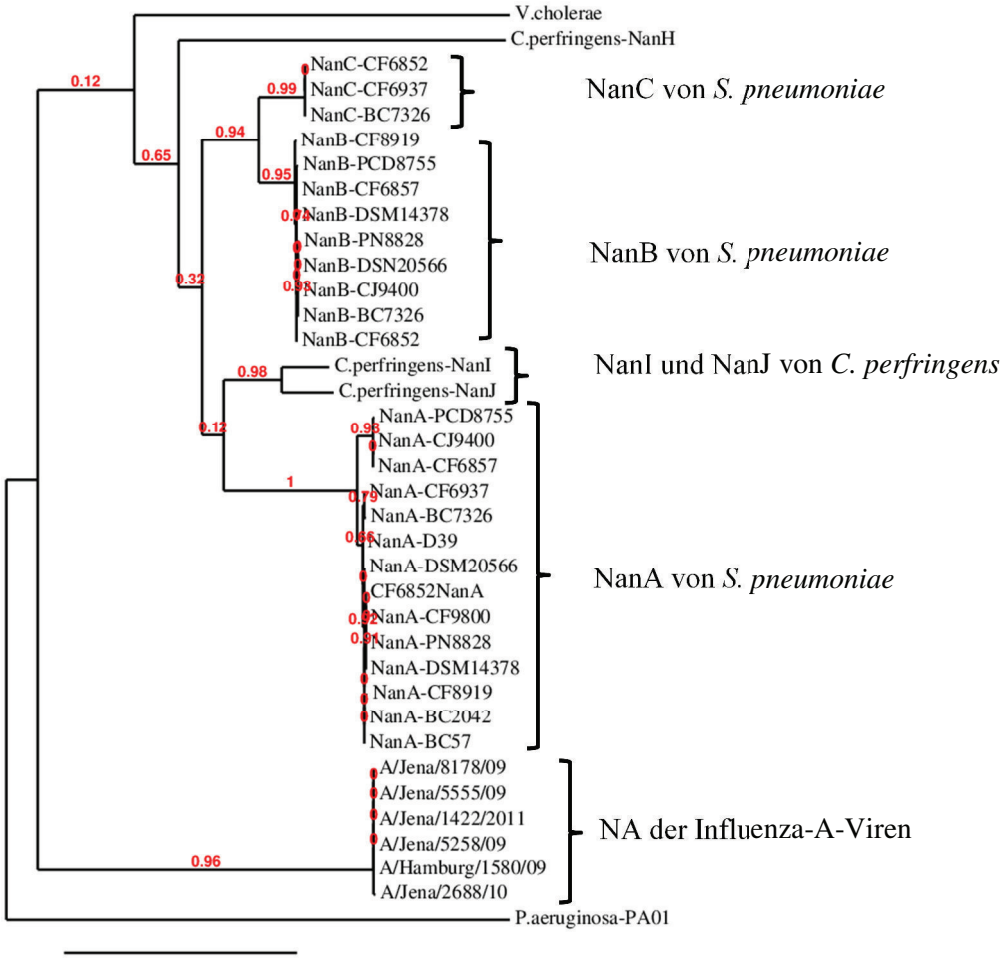

Abbildung 5: Phylogenetischer Stammbaum von viralen und bakteriellen Neuraminidasen (NA). Für das Alignment der Aminosäuresequenzen der NA von fünf Influenza-A-Viren, drei *Clostridium* (*C.*) *perfringens*, je ein NA von *Pseudomonas* (*P.*) *aeruginosa* und *Vibrio (V.) cholerae* sowie 14 NanA, neun NanB und drei NanC von *Streptococcus* (*S.*) *pneumoniae* wurde das Programm Muscle 3.8.31 (Edgar, 2004), für die Stammbaumberechnung PhyML 3.1/3.0 aLRT (Guindon *et al.*, 2010) und für die Darstellung TreeDyn 198.3 (Chevenet *et al.*, 2006) verwendet.

Trotz der Unterschiede in der Primärstruktur teilen alle Sialidasen eine gemeinsame Grundstruktur: ein sechsblättriges β-Propeller-Faltblatt, welches mit den konservierten Aminosäuren das katalytische Zentrum bildet (Taylor, 1996). Dies lässt sich an den Kristallstrukturen der viralen NA (Grienke *et al.*, 2012; Varghese *et al.*, 1991), der NanA (Gut *et al.*, 2011; Xu *et al.*, 2008a) und der NanB (Gut *et al.*, 2008; Xu *et al.*, 2008b) erkennen (Abbildung 1, Seite 11). Die bakteriellen NA weisen zwei weitere strukturelle Merkmale in der Primärstruktur auf. Zunächst das RIP-Motiv, welches aus den Aminosäuren Arginin (R), Isoleucin (I) und Prolin (P) besteht und damit wichtiger Bestandteil der Arginintriade ist. Diese ist für die katalytische Aktivität verantwortlich, da sie mit der Carboxylgruppe der Sialinsäure reagiert. Die Funktion des zweiten Motivs, der Asparaginsäurebox

(Ser/Thr-X-Asp-X-Gly-X-Thr-Trp/Phe), konnte bisher nicht aufgeklärt werden (Newstead *et al.*, 2008). Diese genetischen Charakteristika ließen sich auch in den in Tabelle 2 aufgeführten klinischen Isolaten nachweisen.

Neben der universellen Expression bildet die Konserviertheit des katalytischen Zentrums eine Grundlage für die Wirkung von NAI (Tabelle 3). Mit Ausnahme der Aminosäureposition 442 tragen alle Isolate die identischen Aminosäuren, die für die enzymatische Aktivität essenziell sind. Der Austausch von Isoleucin zu Valin an der Stelle 442 bei drei Isolaten bewirkte jedoch keinen Aktivitätsverlust in den Testsystemen. Die Aminosäuren des aktiven Zentrums der NanB sind ohne Ausnahme hochkonserviert (nicht dargestellt). Ausführlichere Analysen und weitere Experimente zum Einfluss der Aminosäuren auf die Aktivität sowie die Wirkung der NAI finden sich in der Publikation „Sequence diversity of NanA manifests itself in distinct enzyme kinetics and inhibitor susceptibility“ (Xu *et al.* in Vorbereitung).

Tabelle 3: Aminosäuren des katalytischen Zentrums der NanA von den untersuchten klinischen Isolaten. Die drei Arginine (R) der Arginintriade sind hervorgehoben.

	Aminosäurepositionen des aktiven Zentrums von NanA													
	347	366	372	417	442	443	565	590	602	647	**663**	695	**721**	752
DSM20566	**R**	R	D	D	I	F	F	Y	Q	E	**R**	Y	**R**	Y
DSM14378	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D39	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BC2042	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BC57	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BC7326	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CF6852	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CF6937	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CF8919	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CF9800	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CF6857	-	-	-	-	V	-	-	-	-	-	-	-	-	-
PCD8755	-	-	-	-	V	-	-	-	-	-	-	-	-	-
CJ9400	-	-	-	-	V	-	-	-	-	-	-	-	-	-
PN8828	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Funktion der bakteriellen NA im Synergismus

Alle NA besitzen die Funktion, Sialinsäuren von Zelloberflächen abzuschneiden. Bei den Viren leitet dies die Virusfreisetzung ein (Nayak *et al.*, 2009). Im Fall der Pneumokokken entstehen dadurch unter anderem kryptische Rezeptoren für die Anheftung der Bakterien (Plotkowski *et al.*, 1986; Siegel *et al.*, 2014; Tong *et al.*, 2002). Die in der Publikation 3 zusammengefassten Ergebnisse bestätigten, dass rNanA sowohl 2,3- als auch 2,6-verknüpfte Sialinsäuren abspalten kann, während rNanB hauptsächlich die α2,6-Sialinsäure-

Verknüpfungen der Galaktose auf den Zellen spaltet. Damit stimmen NanA und die virale NA bezüglich der Substratspezifität überein, wie bereits publiziert (Gut *et al.*, 2008; Xu *et al.*, 2011).

Vorangegangene Forschungen belegten, dass die Schwere der bakteriellen Sekundärinfektion von der viralen NA-Aktivität abhängt (Alymova *et al.*, 2005; McCullers *et al.*, 2003; McCullers, 2004; Peltola *et al.*, 2005; Peltola *et al.*, 2006). NA vom N2-Subtyp haben eine höhere Aktivität (Peltola *et al.*, 2004), womit sich gut erklären lässt, warum in den letzten drei Jahrzehnten vor allem die H3N2-Epidemien mit erhöhter bakterieller Pneumonie in Verbindung stehen (Glezen, 1982; Simonsen *et al.*, 1997; Wright *et al.*, 1980). In dieser Dissertation wurde hingegen ein *In-vitro*-Modell entwickelt, welches den Einfluss der NA von Pneumokokken auf die Virusausbreitung und den -ertrag verdeutlicht (Publikation 3). Im Modell wurden Lungenepithelzellen (A549-Zellen) mit dem pandemischen H1N1-Influenzavirus Jena/8178 in An- und Abwesenheit von rNanA oder rNanB infiziert. Die Ergebnisse der Virustiterbestimmung mittels Plaquetest und der Untersuchungen zur Virusausbreitung mittels immunohistochemischem Nachweis von viralem NP in den fixierten Zellen nach 48 h belegten, dass beide bakteriellen NA die Virusausbreitung und so den Virustiter erhöhen können. Allerdings verdeutlichten die Resultate auch einen hemmenden Effekt der NA auf die Virusreplikation bei Zugabe von hohen NanA- und NanB-Konzentrationen. Dieser lässt sich auf die massive Abspaltung von Sialinsäuren zurückführen. Letztere werden von den pandemischen Viren genutzt, um mittels HA an die Wirtszellen zu binden und die Virusvermehrung zu initiieren (Childs *et al.*, 2009). Da das HA des Virus nicht mehr an neue Zellen andocken kann, findet nur eine sehr reduzierte bzw. keine Vermehrung in den A549-Zellen statt. Der Virustiter ist infolgedessen mit NanA oder NanB geringer als ohne bakterielle NA. Da das HA der pandemischen Influenza-Isolate von 2009 eine Präferenz für α2,6-verknüpfte Sialinsäuren besitzt (Childs *et al.*, 2009), ist der negative Einfluss der NanA größer als bei NanB. Bei aviären Viren, deren HA bevorzugt an α2,3-verknüpfte Sialinsäuren bindet (Stevens *et al.*, 2006), könnte NanB dagegen von größerer Bedeutung sein.

Mit diesem Modell wurde bewiesen, dass beide NA einen Anteil an der erhöhten Virusreplikation *in vitro* haben und somit zum letalen Synergismus *in vivo* beitragen könnten. Allerdings konnte auch bei einem Überangebot an bakterieller NA ein hemmender Einfluss auf die Virusreplikation verdeutlicht werden.

4.2 Potenzial von neuen NAI in der Therapie des viral-bakteriellen Synergismus

Hemmung der bakteriellen NA durch virale NA-Inhibitoren

Basierend auf der strukturellen und funktionellen Ähnlichkeit von viraler- und Pneumokokken-NA wurden nun die Wirkungen bekannter viraler NAI, wie Oseltamivir, Zanamivir und DANA, zunächst in zwei kommerziellen Testsystemen (FL- und CL-Test) überprüft (Publikation 1, Publikation 2) und dabei publizierte Daten bestätigt (Gut *et al.*, 2011). Beide Testsysteme beruhen darauf, dass durch die enzymatische Aktivität der NA ein künstliches Substrat gespalten wird, wodurch ein FL- oder CL-Signal entsteht.

Sowohl im FL- als auch im CL-Test können Signalinterferenzen auftreten (Publikation 1, Kongkamnerd *et al.*, 2011). Dies zeigte sich beispielsweise im FL-Test bei Katsumadain A (Publikation 1, Publikation 2). Hier war zu erkennen, dass die Werte aus dem FL-Test im Vergleich zu jenen aus dem CL-Test bei allen NA um das Zehnfache erhöht sind. Diese Unterschiede ließen sich auf die Eigenfluoreszenz von Katsumadain A zurückführen, welche das eigentliche Signal überdeckte. Aufgrund dieser hohen Fehleranfälligkeit wurde von Martina Richter ein neues Testsystem entwickelt, welches auf der Hämagglutination von Erythrozyten basiert (Publikation 1). Die Vorteile dieses Testsystems gegenüber dem FL- und CL-Test bestehen darin, dass er unter physiologischen Bedingungen (neutraler pH-Wert, niedrige Salzkonzentrationen) und unter der Verwendung eines natürlichen Substrats (Sialinsäuren auf der Erythrozytenoberfläche) durchgeführt wird. Mit diesem Assay lassen sich sowohl virale als auch bakterielle NA testen. Allerdings ist der HA-HT im Gegensatz zum FL- und CL-Test nicht kommerziell erhältlich und erfordert ein hohes Maß an Expertise.

Zu Beginn der Untersuchung der Pneumokokken-NA wurde nicht zwischen NanA und NanB unterschieden, sondern präzipitiertes Protein des Pneumokokken-Referenzstammes DSM205666 (Publikation 1) bzw. von zwei weiteren Referenzstämmen und sechs klinischen Isolaten (Publikation 2) verwendet. Die NA-Aktivität im Präzipitat aller Isolate ließ sich durch Oseltamivir und DANA (ein Vorläufer von Zanamivir) hemmen. Im weiteren Verlauf der Dissertation wurde die NAI-Empfindlichkeit von rNanA und rNanB des Referenzstamms DSM20566 im FL-Test und im HA-HT überprüft (Publikation 3). Im Vergleich zur viralen NA war die Oseltamivir-Wirkung bei NanA ca. tausendfach vermindert. Die Kinetik mit MUNANA (2′-4-Methylumbelliferyl-α-D-*N*-Acetylneuraminsäure), dem

künstlichen Substrat des FL-Tests, ergab, dass Oseltamivir, wie bei der viralen NA, kompetitiv in das aktive Zentrum der NanA bindet. NanB wies hingegen nur eine schwache Oseltamivir-Empfindlichkeit auf (IC_{50} ca. 80 µM). Dieser Unterschied in der Wirksamkeit von Oseltamivir liegt in der NanA- und NanB-Struktur begründet. Oseltamivir bindet im aktiven Zentrum der Influenzavirus-NA an Glu276, welches mit Arg224 eine Salzbrücke bindet. Dadurch wird eine unpolare Oberfläche für eine Seitenkette des NAI gebildet, die keine enzymatische Spaltung mehr ermöglicht. NanA fehlen vergleichbare Aminosäurereste (Gut *et al.*, 2011). NanB kann nur gering mit Oseltamivir interagieren, weil im aktiven Zentrum alle hydrophoben Reste, die für die Wirkung nötig sind, fehlen (Gut *et al.*, 2011). Zanamivir bindet nicht effizient in das katalytische Zentrum von NanA und NanB, da die für die Interaktion notwendigen Aminosäuren (z. B. Glu119 und Glu227) in bakteriellen NA nicht konserviert sind (Gut *et al.*, 2011).

Kann die bakterielle NA die virale NA in Anwesenheit von NAI ersetzen?

In-vivo-Modelle der Maus zur Untersuchung des letalen Synergismus von Influenzaviren und Pneumokokken wurden bereits in früheren Arbeiten etabliert (McCullers *et al.*, 2001; McCullers *et al.*, 2002). Es konnte gezeigt werden, dass die Behandlung der Influenza mit Oseltamivir eine bakterielle Pneumonie verhindern kann (McCullers *et al.*, 2003; McCullers, 2004). Die Wirkung ließ sich durch die Gabe eines Antibiotikums wie Ampicillin noch verstärken. Die Daten der vorliegenden Dissertation zur Oseltamivir-Empfindlichkeit von NanA ließen vermuten, dass der therapeutische Erfolg von Oseltamivir auf der Hemmung der bakteriellen NA basiert. Um diese Hypothese im *In-vitro*-Koinkubationsmodell zu bestätigen, wurden virus-(Jena/8178)-infizierte A549-Zellen mit NAI in An- und Abwesenheit von rNanA bzw. rNanB behandelt. Hierbei wurde eine Enzymkonzentration eingesetzt, bei der sich der Virustiter verdoppelt, da die Virusausbreitung von den bakteriellen NA unterstützt wurde. Bei der Zugabe von 1 µM Oseltamivir oder Zanamivir ohne bakterielle NA verringerte sich der Virustiter um 99 %. Bei einer Koinkubation von Oseltamivir und rNanA oder rNanB blieb die Virusausbreitung reduziert, was an den wenigen rotgefärbten Zellen im Gegensatz zur Kontrolle ohne NAI in der Immunhistochemie zu erkennen war. Konträr dazu gestaltete sich das Ergebnis mit Zanamivir. Bei Anwesenheit von NanA lag der Virustiter ebenso hoch wie ohne NAI. Daraus folgt, dass NanA die Funktion der NA von Jena/8178 übernehmen kann, wenn diese durch Zanamivir gehemmt ist. In Anwesenheit von NanB war dieser Effekt nicht zu beobachten.

NanB kann die Funktion der viralen NA nicht ersetzen, obwohl NanB wie auch NanA eine Unempfindlichkeit gegenüber Zanamivir aufweist. Diese Unterschiede basieren auf der unterschiedlichen Spaltaktivität von NanA (α2,3- und α2,6-verlinkte Sialinsäuren) und NanB (hauptsächlich α2,3-verlinkte Sialinsäuren). Das HA der pandemischen Viren bindet vorwiegend an α2,6-Verbindungen (Childs *et al.*, 2009). Im Fall von NanB kann das Gleichgewicht zwischen viralem HA und der NA-Funktion nicht wieder hergestellt werden, sodass neugebildete Virionen an der Zellmembran nicht abgeschnitten werden können. Bei aviären Viren, die bevorzugt an α2,3-verknüpfte Sialinsäuren binden, könnte NanB hingegen eine wichtigere Rolle bei der Koinfektion von Viren und Pneumokokken spielen.

In-vivo-Untersuchungen mit einem NA-defizienten Pneumokokkenstamm zeigten, dass die bakterielle NanA den Übergang vom Nasopharynx in die Lunge ermöglicht (Orihuela *et al.*, 2004). Die Gruppe um Jonathan McCullers belegte, dass dieser Funktionsersatz auch mit der viralen NA funktioniert. So kann ein NanA-deletierter Stamm im intranasalen Model in seiner Virulenz geschwächt sein, aber durch vorherige Influenza-Infektion die volle Virulenz wieder erlangen (McCullers, 2006b).

Entwicklung neuer NAI

Durch die verbreitete Unempfindlichkeit von Influenzaviren gegenüber Ionenkanalblockern stellt die einzige erfolgreiche Therapie zurzeit die Behandlung mit NAI dar. Allerdings sind in der Saison 2007/08 auch Virusisolate mit einer Substitution von Histidin zu Tyrosin an der Aminosäureposition 274 (N2-Nummerierung), die eine Oseltamivir-Resistenz vermittelt, aufgetreten (Okomo-Adhiambo *et al.*, 2010). In den letzten Jahren wurden aus diesem Grund viele Studien zu neuen NAI durchgeführt (Hsu *et al.*, 2013; Schade *et al.*, 2014).

Basierend auf der Röntgenkristallstruktur der NA modellieren theoretische Chemiker mögliche Bindungen resistenz-brechender NAI (Grienke *et al.*, 2012). Zusammen mit dem ethnopharmakologischen Ansatz finden sich so neue Leitstrukturen, die in enzym- oder zellbasierten Experimenten untersucht werden können. Oft wird für die erste Prüfung in enzymbasierten Hemmtests auf kommerzielle NA, z. B. von *C. perfringens* oder *Vibrio cholerae* zurückgegriffen. Dabei konnten bereits Naturstoffe aus den Pflanzenwurzeln von beispielweise *Flemingia philippinensis* (Wang *et al.*, 2013), *Glycyrrhiza glabra* (Süßholz, Lakritze; (Grienke *et al.*, 2013)), *Amorpha fruticosa* (Bastardindigo; (Kim *et al.*, 2011))

oder *Sophora flavescens* (Ryu *et al.*, 2008) identifiziert werden. Diese Pflanzen der traditionellen chinesischen Medizin gehören zur Familie der Hülsenfrüchtler, einer Unterfamilie der Schmetterlingsblütler. In den genannten Studien wurden vor allem Flavonoide isoliert, für die eine antivirale Wirkung nachgewiesen wurde. Gerade diese Molekülgruppe verursacht oft falsch-positive Ergebnisse in den enzymbasierten Hemmtests (Chamni *et al.*, 2013; Kongkamnerd *et al.*, 2011), weswegen in dieser Dissertation zur Bestätigung der Wirkung von neuen NAI das Modell der Koinkubation verwendet wurde. So ist es gelungen, NAI zu identifizieren, die als Leitstrukturen im letalen Synergismus von Influenzaviren und Pneumokokken eingesetzt werden können. Sowohl Katsumadain A als auch Artocarpin als bekannte antivirale Substanzen wirkten im niedrigen Mikromolarbereich auf die NanA und NanB der Pneumokokken. Zudem hemmten zwei Azo-Verbindungen (65847 und 73416) die Virusvermehrung und die bakterielle NA-Aktivität. Alle genannten Strukturen verfügen über ein hohes Molekulargewicht und bestehen aus mindestens zwei Ringsystemen und weiteren funktionellen Gruppen, wie Hydroxyl-, Sulfonsäure- oder Azogruppen sowie aus Ether-, Carbonyl-, Methyl- oder Pentylgruppen. Es fällt auf, dass gerade die beiden größten Substanzen aus der Serie der Azo-Verbindungen die niedrigsten inhibitorischen Konzentrationen aufweisen. Die Substanz 65847 besitzt vier Sulfonsäuregruppen und drei Azogruppen. Es kann vermutet werden, dass diese strukturellen Charakteristika die Substanz zu einem universell wirksamen NAI machen.

Zusammenfassend lässt sich sagen, dass nach Ausschluss von Signalinterferenzen alle hier verwendeten Testsysteme vergleichbare Ergebnisse erzielten. In unserem Modell der Koinkubation mit den pandemischen Viren spielte die NanB der Pneumokokken nur eine untergeordnete Rolle, da sie im Gegensatz zu NanA nicht die komplette Funktion der viralen NA übernehmen kann. Aus diesem Grund ist die Wirkung der NAI gegen NanB in diesem Modell von geringer Bedeutung. Nachfolgende Studien sollten auch aviäre Virustypen einschließen, um ein umfassenderes Bild von beiden Pneumokokken-NA im Zusammenspiel mit den Viren bekommen zu können. Die NAI Katsumadain A, 65847 und 73416 besitzen ein breites Wirkungsspektrum mit hohem inhibitorischen Potenzial. Diese können somit als Leitstrukturen für die Entwicklung hochwirksamer NAI zur Überwindung des letalen Synergismus von Influenzaviren und Pneumokokken dienen.

4.3 Die bakteriellen NA als Virulenzfaktor

Bakterielle NA spielen eine wichtige Rolle für die Virulenz der Bakterien. In verschiedenen Studien konnte der Einfluss von NanA auf die Biofilmbildung der Pneumokokken (Oggioni *et al.*, 2006; Parker *et al.*, 2009; Trappetti *et al.*, 2009) und auf das Adhärenzverhalten an Wirtsepithelzellen (Song *et al.*, 2008) nachgewiesen werden. NanB ist weniger gut untersucht, aber auch hier weisen die Publikation auf eine wichtige Funktion als Virulenzfaktor in der Pathogenese hin (Burnaugh *et al.*, 2008; Manco *et al.*, 2006; Muñoz-Elías *et al.*, 2008).

In der vorliegenden Dissertation stellte sich Artocarpin sowohl als Inhibitor der NA als auch als Wirkstoff gegen das planktonische Wachstum, die Biofilmbildung sowie gegen Adhärenz an Lungenepithelzellen heraus (Publikation 2). Allerdings hemmten Oseltamivir, DANA und Katsumadain A trotz inhibitorischer NA-Aktivität weder Wachstum noch Biofilm oder Adhärenz. Hier stellt sich nun die Frage, ob die Bindung der genannten NAI an die NA zu schwach ist, um mit einem natürlichen Substrat zu konkurrieren oder ob die Blockierung der NA keinen Effekt im biologischen System der Pneumokokken hat. Letzteres würde einigen Publikationen widersprechen (Brittan *et al.*, 2012; Parker *et al.*, 2009), aber mit den Studien von King übereinstimmen (King *et al.*, 2004; King *et al.*, 2006). Diese Forschungsgruppe konnte im Vergleich von Wildtypstamm und NA-deletierter Mutante ebenso keinen Unterschied in der Adhärenz an Lungenepithelzellen und bei der Kolonisierung der Mauslunge ermitteln. Mögliche Gründe für diese gegensätzlichen Ergebnisse liegen zum einen in der Verwendung verschiedener Mausmodelle und zum anderen in der Nutzung von Pneumokokken-Stämmen unterschiedlicher Serotypen mit bzw. ohne Kapsel. In dieser Arbeit wurde u.a. ein Pneumokokkenstamm verwendet (CF6937), der kein Gen für NanB aufweist. Jedoch ließ sich bei CF6937 im Vergleich zu den anderen Isolaten kein Unterschied im Wachstum, in der Biofilmbildung oder in der Adhärenz bzw. im Einfluss der NAI nachweisen. Dies weist darauf hin, dass NanB unter den angewendeten Testbedingungen keinen messbaren Einfluss auf den Phänotyp hat.

Im Gegensatz zu vielen anderen Studien (Brittan *et al.*, 2012; LeMessurier *et al.*, 2006; Manco *et al.*, 2006; Parker *et al.*, 2009; Trappetti *et al.*, 2009) standen in dieser Dissertation nicht nur der Pneumokokken-Referenzstamm D39 sondern auch klinische Isolate im Fokus. Der Referenzstamm D39 wurde 2007 von Lanie *et al.* sequenziert (Lanie *et al.*, 2007) und wird häufig in *In-vivo*-Modellen verwendet, da er invasive Infektionen wie z. B. Meningitis oder Sepsis hervorruft. Bei den *In-vitro*-Experimenten stehen andere Eigen-

schaften der Stämme im Vordergrund. So besitzt der Referenzstamm D39 eine Kapsel, wodurch er nur vermindert an Epithelzellen adhäriert (Adamou *et al.*, 1998) und einen schwachen Biofilm ausbildet (Moscoso *et al.*, 2006). Unverkapselte Stämme tragen auf ihrer Oberfläche Adhäsine, die in der Regel von der Kapsel abgedeckt werden. Dadurch gelingt es den unverkapselten Stämmen besser an Zellen zu adsorbieren und sich effektiver untereinander zu vernetzen. Im Fall der Maus ist die Kapsel von Vorteil, da sie vor der komplement-vermittelten Phagozytose schützt (Hammerschmidt *et al.*, 2005; Magee *et al.*, 2001). Diese Unterschiede müssen in Betracht gezogen werden, wenn *In-vitro*-Versuche durchgeführt werden. In anschließenden Arbeiten sollten auch unverkapselte Stämme involviert werden, um den Einfluss der Kapsel ausschließen zu können. Allerdings werden in der Diagnostik nur 0,5 bis 2 % unverkapselte Stämme isoliert (Carvalho *et al.*, 2003), was die Bedeutung der Kapsel aufwertet.

Der Einfluss der NAI wurde in dieser Arbeit nur bei verkapselten klinischen Stämmen getestet. Allein Artocarpin konnte zusätzlich zur NA-Inhibition auch das Wachstum, die Biofilmbildung und die Adhärenz hemmen. Ähnlich wie bei dem Antibiotikum Rifampicin wurde ein bakterizider Effekt festgestellt. Dies bedeutet, dass Artocarpin nach 18-stündiger Inkubation im Konzentrationsbereich von 3 µM die Pneumokokken abtötet. Die Azo-Verbindungen 73416 und 65847, die auch die NA im niedrigen Mikromolarbereich hemmten, konnten nur das planktonische Wachstum, allerdings nicht die Biofilmbildung einschränken. Hier konnten keine bakteriziden Effekte ermittelt werden; das heißt, die Pneumokokken sind in ihrem Wachstum in Anwesenheit des Inhibitors gehemmt und werden nicht abgetötet. Im Allgemeinen bilden sich bei bakteriostatischen Wirkstoffen seltener Resistenzen aus und die Entzündungsreaktion des Wirts ist geringer, weswegen diese Art der Hemmung abhängig von der Art der Therapie oft bevorzugt wird (Pankey *et al.*, 2004).

Aufgrund der ausbleibenden Wirkung von Oseltamivir auf die Biofilmbildung sowie das planktonischen Wachstums ist nicht auszuschließen, dass Artocarpin und die Azo-Verbindungen nicht nur die NA als Zielprotein haben, sondern auch noch mit anderen bakteriellen Proteinen interagieren. Als potenzielle Ziele könnten die Zellwand oder der Replikationszyklus der Bakterien gelten (Kohanski *et al.*, 2010). Um dem letalen Synergismus von Influenzaviren und Pneumokokken vorzubeugen, könnte auf eine Kombination von „echten“ NAI wie Oseltamivir und Artocarpin-ähnlichen Substanzen mit antibakterieller Wirkung gesetzt werden.

5 Zusammenfassung

Sir William Osler bezeichnete *Streptococcus pneumoniae* vor fast einem Jahrhundert als „the captain of all the men of death“ (Watson *et al.*, 1993). Diese Aussage bewahrt auch heute noch ihre Gültigkeit, denn weltweit sterben jährlich 1,6 Millionen Menschen an Pneumokokken-Infektionen (WHO, 2005b). In Kombination mit Influenzaviren steigt die Morbiditäts- und Mortalitätsrate weiter an (Short *et al.*, 2012). Über die Bedeutung der Neuraminidase (NA) als bakterieller Virulenzfaktor für diesen letalen Synergismus ist bisher wenig bekannt. Aus diesem Grund befasst sich die Dissertation intensiv mit der Rolle der bakteriellen NA im letalen Synergismus von Influenzaviren und Pneumokokken und prüft die Eignung der NA als Target für die Therapie mit NA-Inhibitoren (NAI).

Die Eignung der bakteriellen NA als therapeutisches Target wurde bestätigt. Es zeigte sich, dass alle untersuchten klinischen Isolate NanA und zu 96 % NanB exprimieren. Beide katalytischen Zentren sind zudem stark konserviert. Neben dem genetischen Nachweis ließ sich auch die Enzymaktivität in fluoreszenz- und chemilumineszenz-basierten Tests ermitteln, was enzymbasierte Hemmtests mit bekannten (Oseltamivir, Zanamivir) und neuen (Artocarpin, Katsumadain A) NAI ermöglichte. Weiterhin wurden die NAI hinsichtlich ihrer Wirkung auf das Bakterienwachstum, die Biofilmbildung und die Adhärenz untersucht. Die Experimente zeigten eine 25-fach höhere Oseltamivir-Empfindlichkeit der NanA im Vergleich zu NanB. Darüber hinaus wiesen beide NA eine Resistenz gegenüber Zanamivir auf. Artocarpin und Katsumadain A inhibierten beide NA gleichermaßen. In den antibakteriellen Untersuchungen wirkte jedoch nur Artocarpin. Dies lässt vermuten, dass die NA-Hemmung nicht die alleinige Ursache für die antibiotische Wirkung darstellt.

Im Verlauf der Arbeit wurde die neue Wirkstoffklasse der Azo-Verbindungen durch Computermodellierungen von Kooperationspartnern als potenzielle NAI identifiziert. Von insgesamt 18 Substanzen inhibierten fünf sowohl die Virusreplikation als auch die bakterielle NA. Zusätzlich konnte ein leichter Effekt der Substanzen auf das Bakterienwachstum und die Biofilmbildung ermittelt werden.

Um die Rolle der bakteriellen NA im letalen Synergismus mit Influenzaviren zu simulieren, wurde ein *In-vitro*-Koinkubationsmodell mit A/Jena/8178/09 in A549-Lungenepithelzellen etabliert. Damit ließ sich der Einfluss der bakteriellen NA auf die Virusreplikation und -ausbreitung bewerten und die Wirkung neuer NAI testen. Die rekombinant herge-

stellten NanA und NanB erhöhten den Virusertrag sowie die virale Ausbreitung, d. h. sie unterstützten die virale NA beim „Abschneiden“ der Viruspartikel. Bei Zugabe von Oseltamivir ergab sich eine Reduzierung des viralen Titers und der Virusausbreitung sowohl in Ab- als auch in Anwesenheit beider NA. Im Fall von Zanamivir hingegen war dieser Hemmeffekt bei NanA nicht zu ermitteln. Daraus folgt, dass NanA die Funktion der viralen NA übernehmen kann, wenn diese durch Zanamivir blockiert ist. Im Gegensatz dazu wurde die Virusausbreitung auch in Anwesenheit von NanB durch Zanamivir blockiert. Dies bedeutet, dass nur NanA und nicht NanB die Funktion der viralen NA bei humanen Viren übernehmen kann. Die Ergebnisse von immunohistochemischen Untersuchungen zum Nachweis der viralen Rezeptoren (α2,6- und α2,3-verknüpfte Sialinsäurereste) bestätigten zudem, dass die NanB vorwiegend α2,3- und NanA zusätzlich α2,6-verknüpfte Sialinsäurereste enzymatisch spaltet. An α2,6-Verknüpfungen bindet das virale Hämagglutinin (HA) der Influenza-A-Viren der Pandemie von 2009 bevorzugt. Um eine stabile HA-NA-Balance zu gewährleisten, spaltet die virale NA überwiegend α2,6-Verbindungen. Die Spaltfunktion der NanA ähnelt im Gegensatz zur NanB der viralen NA und ist aus diesem Grund in der Lage die Funktion der viralen NA zu ersetzen.

Abschließend wurden die neuen NAI im Koinkubationsmodell getestet. Dabei stellten sich Katsumadain A, Artocarpin und zwei der Azo-Verbindung auch in Anwesenheit von NanA als sehr wirksam heraus. Sowohl der Virusertrag als auch die Virusausbreitung wurden inhibiert. Das *In-vitro*-Koinkubationsmodell eignet sich somit sehr gut, um neue NAI zu identifizieren, die gegen virale und bakterielle NA wirken. Mit diesen Substanzen könnten sich bakterielle Sekundärinfektionen besser bekämpfen lassen.

6 Summary

A century ago Sir William Osler called *Streptococcus pneumoniae* as “the captain of all the men of death” (Watson *et al.*, 1993). This statement is still true because 1.6 million people die due to pneumococcal infection annually (WHO, 2005b). Together with influenza viruses the mortality and morbidity is even ascending (Short *et al.*, 2012). However, the importance of virulence factors like neuraminidase (NA) on the lethal synergism is less investigated. For that reason, the dissertation will focus on the role of bacterial NA in this lethal synergism of influenza viruses and pneumococci. Furthermore, the potential of NA as target for the therapy with NA inhibitors (NAIs) will be clarified.

Bacterial NAs are valid therapeutic targets. We have shown that all clinical isolates under investigation expressed NanA and 96% of them NanB. In addition, the catalytic site is conserved. Beside from the genetic proof, the enzyme activity was demonstrated in fluorescence- and chemiluminescence-based assays. We established enzyme-based inhibition assays with known (oseltamivir, zanamivir) and novel (artocarpin, katsumadain A) NAIs. Furthermore, the NAIs were tested for their inhibition effect on bacterial growth, biofilm formation and adherence. The experiments demonstrated a 25-times enhanced effect with oseltamivir for NanA compared to NanB. Both NAs showed resistance against zanamivir. Artocarpin and katsumadain A inhibited both NAs in a similar concentration range. In the antibacterial investigations only artocarpin were effective. Hence, we suggest that the NA inhibition was not the exclusive reason for the antibiotic effect.

Another scope of the work was the characterization of novel NAIs. Through computer modelling our cooperation partners identified 18 potential NAIs. Five inhibited the viral replication as well as the bacterial NA. Additionally, a slight inhibiting effect of the compounds was observed on bacterial growth and biofilm formation.

In order to simulate the role of the bacterial NA in the lethal synergism with influenza virus an *in vitro* coincubation model was established with A/Jena/8178/09 in A549 lung epithelial cells. We were able to show the influence of the bacterial NA on virus replication and spread. Subsequently, the effect of NAIs was investigated. Recombinant NanA and NanB enhanced the viral yield and spread. Both NAs supported the cleavage function of the viral NA to release new virus particles. By adding oseltamivir viral titres and viral spread were reduced in absence and presence of bacterial NA. In contrast, zanamivir was

not able to inhibit the viral yield in the presence of NanA. Consequently, NanA was able to replace the function of the viral NA in the case of blocking by zanamivir. In the presence of NanB the viral spread was inhibited by zanamivir as opposed to NanA. In conclusion, only NanA was able to substitute the function of the viral NA of human influenza viruses. The results from the immunohistochemical staining to detect the viral receptors (α2,6- und α2,3-linked sialic acids) confirmed that NanB cleaves preferentially on α2,3- and NanA additionally on α2,6-linked sialic acids. The hemagglutinin (HA) of the human influenza A virus binds preferentially on α2,6-linkages. To achieve a HA-NA balance, the viral NA will cleave predominantly on α2,6-linkages. The cleavage function of NanA resembles the viral one and is therefore capable of functional replacement.

Finally, novel NAIs were tested using the coincubation model. Katsumadain A, artocarpin and azo compounds showed strong activity in presence of NanA. Both virus yield and spread were reduced. The *in vitro* coincubation model seems suitable to identify novel NAI inhibiting both viral and bacterial NA. Such compounds could be valid therapeutics to treat secondary bacterial infections.

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8 Eigenständigkeitserklärung

Hiermit versichere ich, dass ich die Promotionsordnung eingehalten habe. Die vorliegende Dissertation wurde von mir selbst angefertigt. Ich habe keine Textabschnitte eines Dritten ohne Kenntlichmachung des Zitats übernommen. Weiterhin habe ich alle von mir eingesetzten Hilfsmittel, persönlichen Mitteilungen und Quellen angegeben.

Jena, den ……..……. ……..

Elisabeth Walther

9 Tabellarischer Lebenslauf

Persönliche Daten

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Promotion und beruflicher Werdegang

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01/2015 bis 03/2015	Promotionsabschluss-Stipendium der FSU Jena
03/2012 bis 12/2014	Wissenschaftliche Mitarbeiter, Universitätsklinikum Jena, Institut für Virologie und Antivirale Therapie
07/2009 bis 03/2011	wissenschaftliche Hilfskraft, Hans-Knöll-Institut, Abteilung für Zell- und Molekularbiologie
07/2008 bis 10/2008	Industrie-Praktikum, Recopharma AB, Huddinge, Schweden

Bildungsweg

04/2011 bis 10/2011	Masterarbeit, Universität Zürich, Vetsuisse Fakultät, Institut für Veterinärpathologie und Hans-Knöll-Institut, Abteilung für Zell- und Molekularbiologie, Note: 1,0
10/2009 bis 03/2012	Pharma-Biotechnologie-Studium, Ernst-Abbe-Hochschule Jena, Abschluss: Master of Science, Abschlussnote: 1,4
03/2009 bis 06/2009	Bachelorarbeit, Hans-Knöll-Institut, Abteilung für Zell- und Molekularbiologie, Note: 1,0
10/2006 bis 07/2009	Biotechnologie-Studium, Ernst-Abbe-Hochschule Jena, Abschluss: Bachelor of Engineering, Abschlussnote: 2,1
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Publikationsliste

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06/2015 **Walther, E.**, Xu, Z., Richter, M., Kirchmair, J., Grienke, U., Rollinger, J. M., Krumbholz, A., Sauerbrei, A., Saluz, H. P., Pfister, W. und Schmidtke, M. (2015). Pneumococcal neuraminidases as key players in the lethal synergism with influenza viruses. *Host Cell and Microbe* (submitted)

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*authors contributed equally to work

09/2011 Vaughan, L., Fehr, A., **Walther, E.** und Schmidt-Posthaus, H. Epitheliocystis and the importance of Chlamydia-related bacteria on fish health. In *Free-living amoebae: an evolutionary crib for emerging pathogens*. (ed. G. Greub)

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Postervorstellungen

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09/2014 **Walther, E.**, Richter, M., Xu, Z., Bohn, K., Rollinger, J.M., von Grafenstein, S., Liedl, K. R., Kirchmair, J., Sauerbrei, A., Pfister, W. und Schmidt-

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03/2014 Richter, M., **Walther, E.**, Bohn, K., Xu, Z., Braun, H., Grienke, U., Rollinger, J. M., von Grafenstein, S., Liedl, K. R., Kirchmair, J., Sauerbrei, A. und Schmidtke, M. Reliability of different assays for identification of novel NAI. 6. - 29.03.2014 24th Annual meeting of the Society for Virology Alpbach.

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09/2013 **Walther, E.**, Xu, Z., Leitzinger, C., Rollinger, J.M., Grienke, U., Kirchmair, J., Liedl, K.R., von Grafenstein, S., Sauerbrei, A., Pfister, W. und Schmidtke, M. Characterization of pneumococcal neuraminidase (NA) and the effect of neuraminidase inhibitors (NAIs) on NA activity, bacterial growth, and biofilm formation. 22-25.09.2013 65. Jahrestagung der DGHM Rostock.

03/2013 **Walther, E.**, Richter, M., Braun, H., Pfister, W., Sauerbrei, A. und Schmidtke, M. Pneumococcal neuraminidases – targets for influenza virus-specific neuraminidase inhibitors (NAI) 06.-09.03.2013 23rd Annual meeting of the Society for Virology Kiel.

10 Angaben zum Eigenanteil

Alle angegebenen Tabellen und Abbildungen finden sich in den jeweiligen Publikationen.

Publikation 1

Titel: Complementary assays helping to overcome challenges for identifying neuraminidase inhibitors

Autoren: Martina Richter, Lilia Schumann, Elisabeth Walther, Anja Hoffmann, Heike Braun, Ulrike Grienke, Judith M. Rollinger , Susanne von Grafenstein, Klaus R. Liedl, Johannes Kirchmair, Peter Wutzler, Andreas Sauerbrei, Michaela Schmidtke

Beitrag zur Publikation: Für diese Publikation stellte ich das Pneumokokken-Präzipitat für die Untersuchungen im FL-Test her. Außerdem führte ich den FL-Test durch (Tabelle 1).

Publikation 2

Titel: Antipneumococcal activity of neuraminidase inhibiting artocarpin

Autoren: Elisabeth Walther, Martina Richter, Zhongli Xu, Christian Kramer, Susanne von Grafenstein, Johannes Kirchmair, Ulrike Grienke, Judith M. Rollinger, Klaus R, Liedl, Hortense Slevogt, Andreas Sauerbrei, Hans Peter Saluz, Wolfgang Pfister, Michaela Schmidtke

Beitrag zur Publikation: In dieser Publikation prüfte ich zunächst, ob die bakterielle NA ein geeignetes therapeutisches Target darstellt. Dazu wurde das bakterielle NA-Gen in allen klinischen Isolaten mittels PCR nachgewiesen und im Anschluss sequenziert (Tabelle 1). Weiterhin ermittelte ich die NA-Aktivität der Pneumokokken-Präzipitate, als auch von der rekombinant hergestellten NanA im FL- und CL-Test. In diesen Testsystemen erfolgte im Folgenden die Prüfung der bekannten NAI Oseltamivir, Zanamivir und DANA sowie der neuen antiviralen Substanzen Artocarpin und Katsumadain A (Abbildung 1). Die Untersuchungen zum NAI-Einfluss auf das Bakterienwachstum, die Biofilmbildung und die Adhärenz von *S. pneumoniae* wurden von mir durchgeführt (Tabelle 3, Abbildung 3 und 4). Die dargestellten zytotoxischen Effekte in Abbildung 5 wurden ebenso von mir ermittelt. Auch an der Verfassung des Manuskripts zur Veröffentlichung habe ich mitgearbeitet.

Publikation 3

Titel: Neuraminidase inhibitors help to overcome the lethal synergism of influenza viruses and pneumococci

Autoren: Elisabeth Walther, Zhongli Xu, Martina Richter, Johannes Kirchmair, Ulrike Grienke, Judith M. Rollinger, Andi Krumbholz, Andreas Sauerbrei, Hans Peter Saluz, Wolfgang Pfister, Michaela Schmidtke

Beitrag zur Publikation: Die Entwicklung des Koinkubationsmodells (Abbildung 3, Tabelle 1) sowie dessen Anwendung (Abbildung 4, Tabelle S2) einschließlich Sialinsäure-Nachweis (Abbildung 2) wurden von mir durchgeführt. Weiterhin erstellte ich die Michaelis-Menten-Kurven (Abbildung 1 und S1) und die Daten zur Enzymaktivität. Darüber hinaus wurden die FL-Daten in Tabelle 2 von mir generiert. Auch an der Verfassung des Manuskripts zur Veröffentlichung habe ich mitgearbeitet.

Die Arbeitsanteile wurden in Inhalt und Umfang richtig ausgewiesen.

…… ……

Prof. Dr. Hans Peter Saluz Prof. Dr. Wolfgang Pfister

11 Danksagung

Diese Promotionsarbeit wurde am Universitätsklinikum Jena am Institut für Virologie und Antivirale Therapie angefertigt.

An erster Stelle geht mein Dank an PD Dr. Michaela Schmidtke für die immerwährende fachliche Unterstützung und die wegweisenden Diskussionen. Darüber hinaus möchte ich mich für ihre Geduld und für ihr positives Denken bedanken. Ich danke ihr außerdem dafür, dass ich an zahlreichen nationalen wie internationalen Tagungen teilnehmen durfte.

Ein besonderer Dank gebührt meinen Doktorvätern Prof. Dr. Hans Peter Saluz und Prof. Dr. Wolfgang Pfister für die fachliche Betreuung, die anregenden Diskussionen und das stetige Interesse am Fortgang der Experimente. Ebenso möchte ich mich bei Prof. Dr. Andreas Sauerbrei als Leiter des Instituts bedanken, der mir die Möglichkeit gab, diese Dissertation in der Virologie anzufertigen.

Einen großen Anteil am Gelingen der Arbeit ist der gesamten Arbeitsgruppe von PD Dr. Michaela Schmidtke zuzuschreiben. Mein Dank geht an Anja Hoffmann, Martina Richter, Birgit Jahn, Karolin Hess, Christin Leitzinger, Dr. Nora Seidel, Dr. Heike Braun und Dr. Zhongli Xu für die Hilfsbereitschaft und die freundliche Atmosphäre. Wir haben viel zusammen diskutiert und gelacht und es hat großen Spaß gemacht, in diesem Team gearbeitet zu haben.

Weiterhin danke ich dem gesamten Institut für die gute Zusammenarbeit, stellvertretend möchte ich hier Dr. Michael Schacke für Bereitstellung der Zellkulturen und die Arbeitsgruppe von Prof. Dr. Roland Zell für die Sequenzierung nennen.

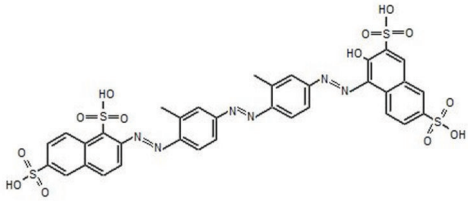
Ein großes Dankeschön geht an die Arbeitsgruppe von Prof. Dr. Judith Rollinger und Prof. Dr. Johannes Kirchmair, die zum einen die Substanzen identifiziert haben und somit die Durchführung der Promotion erst ermöglicht haben. Zum anderen haben sie stets passende Hinweise für die Paper und Konferenzbeiträge gefunden.

An meine Freunde, Eltern und Großeltern geht ein besonders herzliches Dankeschön für die moralische Unterstützung. Zum Abschluss möchte ich mich an dieser Stelle bei meinem Freund Nico Dietrich bedanken. Ohne seine geduldigen Hinweise beim Anhören von Vorträgen oder beim Korrekturlesen wäre die Arbeit nicht zustande gekommen. Ich hoffe ich kann mich in zwei Jahren revanchieren.

12 Anhang

Strukturformeln der Azo-Verbindungen

65847



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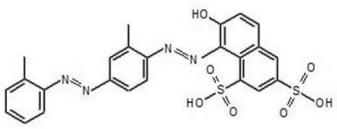

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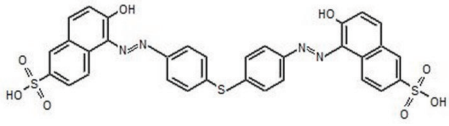

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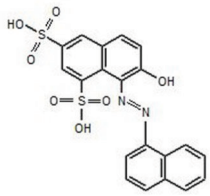

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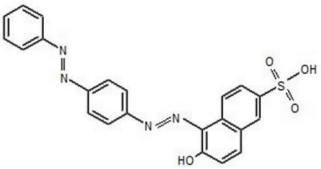

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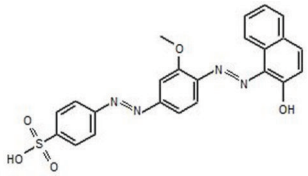

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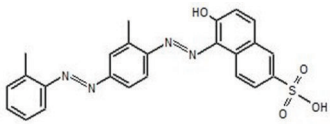

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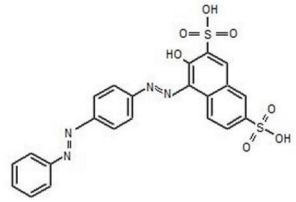

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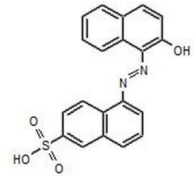

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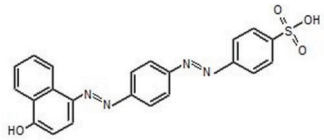

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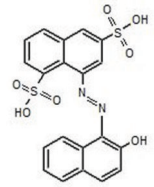

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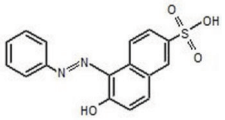

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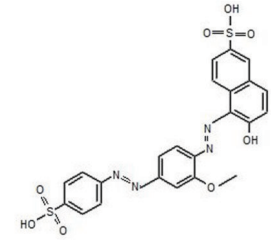

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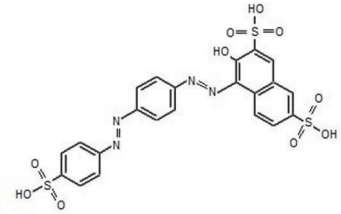

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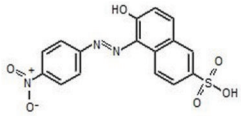

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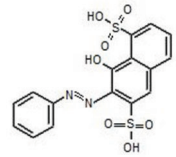

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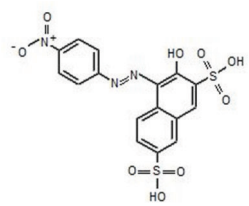

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